Paedogenetic reproduction in Mycophila speyeri and Heteropeza pygmaea (Cecidomyiidae, Diptera)

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Paedogenetic Reproduction in *Mycophila speyeri* and *Heteropeza pygmaea* (Cecidomyiidae, Diptera).

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List of Abbreviations

Å	Angstrom
c.p.m.	counts per minute
GC-MS	gas chromatography-mass spectrophotometry
H.P.L.C.	high perfomance liquid chromatography
i.d.	internal diameter
I.R.S.	inactivated rabbit serum
IGR	insect growth regulators
JH	juvenile hormone
JHa	juvenile hormone analogue
kDa	kiloDaltons
m/z	mass charge
µg	microgram
µl	mcrolitres
µm	micrometres
mM	millimolar
M	molar
ng	nanogram
nm	nanometre
O.D.	optical density
pg	picogram
ppm	parts per million
PTTH	prothoracicotropic hormone
p.s.i.	pounds per square inch
R.E.R.	rough endoplasmic reticulum
R.I.A.	radioimmunoassay
R_T	retention time
SD	standard deviation
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SE	standard error

S.E.R.	smooth endoplasmic reticulum
T.E.M.	transmission electron microscopy
T.I.C.	total ion current
T.L.C.	thin layer chromotography
v/v	volume per volume
w/v	weight per volume

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For Kathleen Mary Inglis

Abstract

A study has been made of paedogenesis in the larvae of *Mycophila speyeri* and *Heteropeza pygmaea* (Cecidomyiidae, Diptera). Both insects were capable of paedogenetic reproduction; they produced live offspring asexually while still in the larval form. Under certain circumstances the larvae would produce offspring that did not reproduce as larvae; these individuals pupated and produced adults. The adults studied were all female; they laid eggs but these did not go on to develop parthogenetically.

Special attention was paid to the conditions that determine whether the larvae of *M. speyeri* reproduce by paedogenesis or whether they pupate and produce adults. A number of different conditions were examined including: temperature, nutrition, fungal species and insect population density. It was found that although these conditions affected the speed of development and the insect's fecundity, no one environmental factor could be isolated as being responsible for a switch between a paedogenetic reproductive cycle and the production of adults.

In an alternative approach to the problem of the control of paedogenesis, the effect of exogenous hormone application was studied. The ecdysone agonists, RH 5849 and RH 5992, did not have an effect on the life cycle of *M. speyeri*. Although the JH analogue, pyriproxyfen, reduced the ability of *M. speyeri* larvae to produce offspring in one experiment these results could not be replicated.

Some insects that eat only fungi have unusual sterol metabolism because fungi do not contain cholesterol. The sterol present in the fungus, *Chondrostereum purpureum*, and therefore the dietary sterol for the mycetophagous Cecidomyiidae studied here, was identified using GC-MS, as ergosterol. *H. pygmaea* tissue however, was found to contain not only ergosterol but also cholesta - 5, 7, 22- 3 β - ol. The presence of cholesta - 5, 7, 22- 3 β - ol suggests that in the absence of dietary cholesterol *H. pygmaea* is able to dealkylate ergosterol in order to produce ecdysone and 20-hydroxyecdysone. The presence of 20-hydroxyecdysone was confirmed by a

radioimmunoassay. However due to the very small amounts of material available for analysis the presence of ecdysone could not be confirmed. The question of the role of ecdysteroid hormones in the control of paedogenesis remains unresolved.

The role of the fat body during paedogenesis in *M. speyeri* was studied using transmission electron microscopy, the analysis of protein samples from larvae of different stages and the *in vitro* culture of fat body lobes. The structure of the maternal fat body varied according to the stage of development of the daughter larvae within the mother larva's body. Lipid droplets accumulated during the early stages but were depleted towards the time at which the daughter larvae would leave the mother. There was evidence of active protein synthesis at all times, but no sign of either the accumulation of stored protein nor the export of protein containing vesicles into the haemolymph.

The study of proteins present in the maternal fat body, maternal haemolymph and growing embryos, however, showed that some fat body protein also appeared in the other tissues. Attempts to follow protein synthesis in cultured fat body cells succeeded in measuring the transfer of proteins from the fat body to the culture medium. The pattern produced appeared to be typical of that of haemolymph proteins, but the amount of protein released did not continue to increase with culture time. In summary, evidence has been gathered for an active transfer of nutrients from maternal to embryonic tissues, and no evidence has been found for the parasitic or predatory "consumption" of the mother by her offspring.

Chapter 1

Introduction: the paedogenetic Cecidomyiidae

1.1 The Cecidomyiidae.

The Cecidomyiidae are lower flies; members of the Order Diptera, Suborder Nematocera. Many Cecidomyiidae form galls on plants, and so members of the family are often known as "Gall Midges". However many cecids have free-living mycetophagous larvae. It is these insects which form the subject of this study.

The family is thought to be made up from three subfamilies: the Cecidomyiinae, the Porricondylinae and the Lestremiinae. The two species of cecid which are the subject of this study are *Mycophila speyeri* from the subfamily Lestremiinae and *Heteropeza pygmaea* from the subfamily Porricondylinae (Mamaev and Krivosheina 1993 and Matuszewski 1982). Like many other free-living cecids, these insects are able to reproduce by the extraordinary mechanism of paedogenesis (i.e. reproduction in the larval or other immature form). The process of paedogenesis is the subject of this thesis. *M. speyeri* and *H. pygmaea* are serious pests of cultivated mushrooms, which is a good reason for studying these insects.

1.2 External morphology of the larvae.

Identification of different species of cecid is usually made at the adult stage. However, from an economic point of view, the important stage in the development of the

Cecidomyiidae is the larval stage. It is this stage which causes the economic damage in phytophagous and mycetophagous species and the stage during which the phenomenon of paedogenesis takes place. Mamaev and Krivosheina (1993) attempt to provide a key to allow identification at the important larval stage.

The larvae consist mainly of a head, neck and 12 segments: 3 thoracic and 9 abdominal. The head always appears to be small in comparison to the rest of the body. It is produced from a fully developed head capsule and has a labrum, a labium, mandibles and maxillae. The antennae are attached to the head capsule and papillae are attached to the dorsal and ventral surfaces of the body (Mamaev and Krivosheina 1993).

Many larvae of the Cecidomyiidae have groups of bristles on the ventral side. These are known as the creeping welts and have a locomotory function. The pattern that these spinules form often plays an important role in the identification of the species at the larval stage (Mamaev and Krivosheina 1993).

The shape and size of the body varies greatly between the different species depending upon the environment in which they live. The larvae which live within decaying wood tend to be cylindrical in shape (Wyatt 1961).

1.3 Gall midge biology - trophic groups.

There are three main trophic groups in the Cecidomyiidae: zoophages, phytophages and mycetophages (Mamaev and Krivosheina 1993). Phytophagous cecids are those

which feed on the living tissue of higher plants. They include many free-living species and many of the gall-forming species which give the family the common name of the gall midges.

One of the best known gall-forming cecids is *Mayetiola destructor*, the hessian fly, which is a serious pest of cultivated cereals. The galls produced on an infested plant are a reaction of the plant to attack by the fly. They benefit the insect by producing a safe and nutritious environment within which it can develop.

The zoophages form a much smaller group in terms of species number than the phytophages. They usually feed on sedentary species such as lice, aphids and scale insects (Mamaev and Krivosheina 1993). They can also be found living as internal parasites of species of aphids and psyllids (Matuszewski 1982).

Mycetophagous cecids live on fungi. They include species that live in the soil, underneath the bark of rotting wood, on fungal fruiting bodies and in the layers of leaf litter on the forest floor. The species found on decaying plant material vary depending upon the degree of decay of the tissue. Species such as *Miastor metraloas* and *H. pygmaea* are characteristically found on wood in an advanced state of decay (Matuszewski 1982).

Mycetophagous Cecidomyiidae, such as the ones in this study, feed from the fungal mycelium by piercing the hyphal walls and sucking out the protoplasm (Camenzind

1962). This group of the Cecidomyiidae is of economic importance as the insects are serious pests of intensively cultivated mushrooms (Anon. 1982).

1.4.1 Mushroom production.

Edible fungi have been cultivated for many centuries. In Great Britain the cultivation of edible mushrooms is one of the largest horticultural industries (Fletcher, White and Gaze 1989). Commercial cultivation of mushrooms in Great Britain, as in many other European countries, has grown rapidly since the Second World War (Wyatt 1960). In 1981 the total U. K. production of mushrooms was over 60 000 tonnes, with the majority of this being grown in the South East of England and in Northern Ireland (Flegg, Spencer and Wood 1985).

The major species of fungus grown for consumption is *Agaricus bisporous*. In its vegetative phase it produces a hyphal mycelium, while in its sexual stage it produces many different spore bearing structures, one of which is the "mushroom" that we eat.

Many different cultivation techniques have been employed over the centuries to cultivate mushrooms for consumption. The following is a description of the techniques used in the British mushroom industry today.

Mushrooms are grown on compost, larger farms will produce their own compost, while smaller farms may buy it ready made. The compost is usually made from wheat straw and poultry compost, poultry compost normally is used as it has a consistent composition (Fletcher *et al.* 1989). These materials are wetted and mixed together

(Flegg *et al.* 1985). The compost is turned and mixed regularly for around 14 days; during this time composting occurs and raises the temperature to around 80 °C. The compost is then packed into long rooms which have slatted floors where it is pasteurised for 4 to 8 days at 50 - 60 °C (Flegg *et al.* 1985). This pasteurisation process kills many of the pests and diseases that would otherwise be contained within the compost.

After cooling the compost is mixed with mushroom spores. Because the spores are very small they are usually introduced to the compost using sterilised cereal grains as a carrier (Flegg *et al.* 1985). The compost is then packed into wooden or metal trays or into plastic sacks, depending on the production system used. The fungal mycelium is allowed to "run" through the compost, at an optimum temperature of 24 °C. This normally takes around 10 days (Flegg *et al.* 1985).

In order to induce fruiting the compost must be covered by what is known as a "casing layer". This layer is made from peat and often includes sphagnum moss and chalk. It is not known why a "casing layer" is needed but, if it is not applied, then the fungus continues to grow vegetatively and does not produce any fruiting bodies (Fletcher *et al.* 1989). After casing the first fruiting bodies are seen after around 20 days.

When fruiting begins the crop is produced in a series of "flushes", each flush is harvested from the crop and there is normally a period of around 7 - 10 days between each flush. The intensity of production and the number of flushes harvested from each crop depend largely on the size of the mushroom farm in question. Farms often stagger

mushroom production to ensure that at any one time there is compost being produced, crops at the various stages of development and that there are always crops producing flushes of mushrooms which can be harvested (Flegg *et al.* 1985).

Within the mushroom houses the temperature and humidity are carefully controlled to provide the optimum conditions for production. Light is not needed for the growth of the mushroom so the houses are normally dark and are only illuminated for the purposes of picking and maintaining the crop.

Shelf systems are used in modern mushroom production in order to maximise the area available in the houses. These can be made from wood but in more modern farms they are made from metal as, although they are more expensive, these trays last longer in the humid conditions needed to produce mushrooms and can be cleaned more thoroughly to maintain levels of hygiene. The metal trays also allow for a degree of mechanisation in the spraying of the crops although harvesting is still done by hand. Many small farms use the "bag" system of production. This allows them to buy their compost, ready inoculated with spores and to produce the mushrooms in the same bags (Fletcher *et al.* 1989). This system avoids the need for the large capital outlay needed to purchase shelves or composting equipment.

1.4.2 Pests of mushroom crops.

Due to the continuous cropping of mushrooms and the controlled conditions within the mushroom houses the crops are prone to infections from viruses and many fungal and bacterial diseases (Wyatt 1960, Flegg *et al.* 1985). These diseases can reduce the yields

obtained and cause the production of imperfect fruiting bodies which are unsuitable for sale.

Mushroom crops are affected by relatively few insect pests, as pasteurisation of the compost normally kills any insects which are contained within the compost. Any pests which are found in the crop usually enter the mushroom houses after the pasteurisation process and are often introduced with the casing layer. Nematodes and mites can infest mushroom crops but the majority of the pests are flies from the families of Phoridae and Sciaridae (Anon. 1982). The phorid larvae cause damage by feeding extensively on the fungal mycelium, while the sciarid larvae have large chewing mouthparts and feed on the small emerging fruiting bodies, known as "pinheads". Pesticides can be used to treat these pests in the compost or the casing layer but some chemicals can have a deleterious effect on the mushroom crop and reduce yields (Hussey, Wyatt and Hughes 1960, Flegg *et al.* 1985, White 1986 and Cantelo 1992). Mushroom farmers are often reluctant to use pesticides and rely on strict hygiene to avoid infestation by pests and diseases.

1.4.3 Cecids as pests of mushrooms.

Many species of Cecidomyiidae have been found to infest commercial mushroom crops. These include *H. pygmaea* and *M. speyeri.* The larvae of these species are obligate mycelial feeders and feed on the growing mycelial front within the compost. They pierce the mycelium with their mouthparts and suck out the protoplasm (Flegg *et al.* 1985). Cecid larvae are thought to be introduced to the crop from the peat present in the casing layer and can be introduced to the mushroom houses on the

clothes of workers and on the trays and shelves, which can be a particular problem if wooden trays are used. (Anon. 1982).

The damage that the cecids cause to the fungal mycelium can cause depression of yield in the crop. They can also act as vectors of viral and bacterial diseases (Flegg *et al.* 1985). The ability of the cecids to reproduce exponentially by paedogenesis means that they can achieve huge populations. When numbers become high they migrate onto the fruiting bodies and cause cosmetic damage to the crop. The cosmetic damage caused by *M. speyeri* larvae is particularly severe because of their orange colour (Figure 1.1).

Flegg *et al.* (1985) estimate that 18 *M. speyeri* larvae present per m^2 during the spore running stage can cause 51% spoilage of the crop. The adult cecid flies are not considered to be a problem as it is the high level of larval reproduction which can make these insects pest species (Anon 1982). White (1990) studied the effect of *H. pygmaea* on the cropping of mushrooms and concluded that the reduction in yield was a problem as well as spoilage. The reduction in yield was greater when the cecid larvae were introduced at the spawning stage rather than at the casing stage. He also found that the loss of yield was greater if the initial infestations were higher.

The control of cecids within the mushroom house is difficult and, as much of the damage is cosmetic, the killing of larvae once they are on the crop is pointless. The larvae of *H. pygmaea* and *M. speyeri* are also difficult to kill using chemical

Figure 1.1 Cultivated mushroom, *Agaricus bisporous*, infested with *Mycophila speyeri*.

pesticides and these chemicals are often used by farmers simply to depress the growth of populations rather than to eradicate the insects (White 1977).

Mushroom farmers rely on cultural techniques to keep the crops free of cecids and to prevent them from spreading around the farm once there has been an outbreak (Anon 1982). These measures include: the use of peat extracted from non-wooded areas, the pasteurisation of compost, the heat treatment of houses after production, the disposal of all vegetative waste, the isolation of any infested houses and foot baths at the entrance of each house.

1.5 Paedogenesis.

In some species of the Cecidomyiidae, such as *M. speyeri* and *H. pygmaea*, the majority of reproduction is viviparous, parthenogenetic and occurs in the larval stage. This process is known as paedogenesis. The first description of paedogenesis was in the cecidomyiidae, *Miastor metraloas* (Wagner 1863).

In cecids paedogenesis involves the precocious development of eggs within the body of the mother and the birth of live young. Ivanov-Kasas (1965) describes it as a form of parasitism within one species. The eggs develop in the female ovary and are released into the body cavity, where they develop and finally emerge from the dead mother (Camenzind 1962). Obviously this is not parasitism in the usually accepted sense, but there is a functional analogy.

This form of reproduction is only found in the mycetophagous species of the subfamilies Porricondylinae and Lestremiinae and is most common in those species which live in rotting wood where there are large amounts of food present (Mamaev and Krivosheina 1993).

The ability to reproduce paedogenetically enables cecids to multiply rapidly and to reach pest status quickly. Cuellar (1977) states that proliferation in a parthenogenetic individual is always more rapid than in sexual forms of the same species, because all of the zygotes produced are egg-producing females, no eggs are wasted on males. Even if the average number of offspring produced is the same, the potential for increase in the population is double that of bisexual species.

Parthenogenesis has another advantage; it enables a single individual to establish a new colony. Parthenogenetically reproducing species are often found in environments with much disturbance like flood planes and areas where regular natural burning occurs (Cuellar 1977, Went 1982), the species is able to rapidly colonise the area while conditions permit.

1.5.1 Paedogenetic reproductive system.

The paedogenetic lifestyle has led to some specialisation of the reproductive system in those cecids that practice it. Paedogenetic cecid larvae have no external genitalia, and there is no genital tract; an ovary is present, however.

Insect ovaries are classified according to the type of follicles they produce. Panoistic ovaries contain ovarioles which have only follicle cells around their oocytes, while meroistic ovaries contain oocytes which are connected by cytoplasmic strands to nurse cells which provide them with proteins and nucleic acids (Nijhout 1994). Most holometabolous insects have polytrophic ovaries; in which the oocytes remain attached to the nurse cells and are surrounded by the follicle cells (Highnam and Hill 1977).

Ovaries in the Cecidomyiidae belong to the meroistic-polytrophic type. They are without ovarioles and genital ducts (Matuszewski 1982, Schüpbach and Camenzind 1983, Schüpbach and Went 1983). The ovaries consists of two groups of egg follicles. Each follicle comprises an oocyte associated with a number of nurse cells surrounded by a follicular epithelium (Went 1982).

Non paedogenetic adult cecid females lay between 1 and 4 eggs. By contrast paedogenetic larvae can have as many as 40 eggs present in the ovaries (Went 1982). Ivanova-Kasas (1965) estimates that in *H. pygmaea* the average number of follicles per insect is 32.

Oogenesis in paedogenetic individuals is very rapid when it is compared to imaginal egg development. The eggs start embryogenesis before they would normally have completed oogenesis (Went 1982). Matuszewski (1982) describes how this can be determined as either a failure in the establishment of a meiotic block or the precocious development of the eggs.

The eggs do not increase in size during oogenesis. The majority of the size increase in the offspring occurs during embryogenesis, while the embryo is within the haemocoel of the mother-larva.

In other species of invertebrates development of the egg is triggered by oviposition or sperm entry. Since paedogenetic eggs are neither laid, passed along ovarian ducts nor fertilised, these stimuli are not available. It is not known what triggers the development of the follicle or the release of eggs into the haemocoel. It could be due to fluctuation in the titres of developmental hormones; this idea is explored in Chapter 4.

Many authors including: Madhavan (1973), Mahowald and Stoiber (1974), Schüpbach and Camenzind (1983) and Schüpbach and Went (1983) have used time lapse photography and transmission electron microscopy to monitor the growth and development of the ovaries and eggs in *M. speyeri* and *H. pygmaea* both *in vitro* and *in vivo*. They describe in detail the stages that occur during this type of development. The following description is based largely on *H. pygmaea*.

1.5.2 Oogenesis.

When paedogenetic cecid embryos are at the blastoderm stage of development, within the body cavity of their mother-larva, 4 pole cells lie at the posterior end and are surrounded by somatic cells. As germ-band formation occurs, the pole cells with their surrounding somatic cells are pushed into the inner part of the embryo by the increasing germ band, eventually reaching the sixth abdominal segment where they continue their development (Panelius 1967). From this point the pole cells are

described as oogonia. There are 2 oogonia along with 30 - 40 mesodermal cells in each of 2 ovaries. Each oogonium passes through 3 mitotic divisions resulting in 16 oogonia per ovary (Madhavan 1973, Matuszewski 1982). At the same time the mesodermal cells divide producing around 500 cells.

1.5.3 Nurse-chamber formation

The oogonia now divide mitotically for the last time; this division is the same as previously except for the fact that the two cells do not separate completely and remain connected as sister cells by a cytoplasmic bridge (Mahowald and Stoiber 1974).

In *H. pygmaea* four mesodermal cells gather around the 2 connected sister cells. These mesodermally derived trophocytes all fuse with the same cell, which becomes a germ line derived trophocyte known as the nurse cell (Mahowald and Stoiber 1974). The other sister cell becomes the oocyte. The function of the nurse cell, or chamber as it is sometimes called, is thought to be the production and supply of RNA to the oocyte (Matuszewski 1968).

The number of mesodermal cells which fuse with the nurse cell can vary depending on the species in question (Mahowald and Stoiber 1974, Matuszewski and Jazdowska-Zagrodzinska 1994).

The formation of a multinuclear nurse chamber as a result of the fusion of a variable number of somatic cells with the germ-line-derived mononuclear nurse cell is prevalent in the whole of the Cecidomyiidae. Matuszewski and Jazdowska-Zagrodzinska (1994)

describe this as being a very important feature which enables large amounts of RNA to be transferred from the nurse chamber to the growing oocyte and state that this is very important in a group of insects which they suggest has very low ribosomal gene content.

Initially it was thought that the nurse cells were entirely somatic in origin (Panelius 1967). It was only later work such as that of Madhavan (1973) that recognised they were of dual origin. The fact that the nurse chamber is not just derived from the oogonium is peculiar to the Cecidomyiidae. In other insects the nurse chambers are almost always derived from the oogonium with no contribution from the mesodermal cells (Madhavan 1973).

The nurse chamber in the Cecidomyiidae is a true syncytium with no membranes remaining between the constituent cells (Schüpbach and Went 1983).

1.5.4 Oocyte-nurse-chamber complex.

At this point in oogenesis the future mother embryo, in which the oocyte-nurse-cell complex is contained, is released from the maternal cuticle and begins its life as an independent larva. Two mesodermal cells from the nurse chamber then fuse with the oocyte (Schüpbach and Went 1983). The nuclei of these 2 somatic, mesodermal cells within the oocyte are known as the somatic ovary nuclei. Schüpbach and Went (1983) describe this spontaneous fusion of somatic cells and germ line cells as being very unusual.

After this fusion many mesodermal cells surround the oocyte-nurse-cell complex and form the follicular epithelium (Camenzind 1982). The follicle now comprises the tri-nucleate oocyte and the nurse chamber surrounded by the follicular epithelium; it is known as the oocyte-nurse chamber complex.

The 2 ovaries in the young *M. speyeri* larvae are now filled with follicles, each containing an oocyte-nurse-chamber complex. The average number of follicles per ovary is 16 but this can vary depending on the nutritive state of the mother larva (Matuszewski 1968, Meats and Tucker 1976, Schüpbach and Camenzind 1983).

Around 20 hours after the larva has hatched from its mother, the sheath surrounding the ovary ruptures and the follicles are released into the haemocoel.

1.5.5 Follicular epithelium.

In most insects the follicular epithelium produces the chorion which protects the egg from the environment after it has been laid. This is not the case in the paedogenetic cecids, in which no chorion is present.

The follicular epithelium is of mesodermal origin as are the nurse cells. The follicle cells are very important in the formation of the egg (Went 1978a, Went and Junquera 1981), since they force the eggs to elongate in a direction parallel to the polar axis.

Once oogenesis is complete the embryos continue to grow in the antero-posterior axis. Went (1978a) succeeded in culturing eggs without a follicular epithelium by irradiating

the ovary or by disrupting the ovary wall before the follicular epithelium had been produced. These eggs grew to the blastoderm stage and were spherical in shape. Work carried out by Junquera (1983a) confirmed that these 'naked' blastoderms did have pole cells. The lack of the follicular epithelium did not appear to have an effect on the location within the embryo of the cells; rather it was the position of the nurse cell which determined their position. In eggs without follicular epithelium the nurse cells degenerated and the eggs failed to develop further.

1.5.6 Embryogenesis.

During normal paedogenetic development, the nurse cells and nutritive chamber degenerate at the end of oogenesis. Once this occurs then nutrition in the growing embryos is linked with nutrient transfer from maternal tissues. A study of this interesting phenomenon is presented in Chapter 5.

The majority of growth occurs during embryogenesis, with the embryos increasing in size by 200 times from the end of oogenesis to when the new larvae emerge from the maternal cuticle (Camenzind 1982). The follicular epithelium becomes very thin as the embryo increases in size (White 1946).

Segmentation during embryonic development in the paedogenetic Cecidomyiidae is typical of the process that occurs in all insects (Ivanova-Kasas 1965). When there have been 8 divisions the cleavage nuclei move to the surface of the egg, their cytoplasm forming a syncytial blastoderm. The division of the blastoderm cells continues synchronously. Only the germ-line cells depart from the rhythm and divide more

slowly. During the formation of the blastoderm nutritive substances ("yolk") accumulate in the central part of the embryo (Ivanova-Kasas 1965). By the time that the segmentation is complete the nutritive chamber has disappeared.

The blastoderm is initially homogenous but rapidly goes on to develop a germ band on the ventral side of the egg. As this spreads the serosa and the amnion are produced. At this point in development the embryo itself develops pole cells which will become the eggs for the next generation.

The lengthening of the germ band is accompanied by its segmentation beginning at the head segment. At this time the migration of cells into the median groove leads to the formation of the mesoderm. During this development the embryo is continuing to grow.

The germ band begins to shorten and the initials of all the organs begin to appear. The accumulation of stored nutrients continues; they are contained within the yolk syncytium and this occupies the dorsal part of the egg.

As the egg grows in size, so the follicular epithelium stretches and becomes thinner. Organs such as the gut and the fat bodies continue to develop. While this development is underway in the embryo changes can be seen in the mother larva. She decreases her food intake and becomes motionless.

1.5.7 Chromosomes.

The Cecidomyiidae are unusual in that they have a different number of chromosomes in their somatic cells from that present in the germ line cells (Kunz, Trepte and Bier 1970). This is true of both paedogenetic and bisexually reproducing species (Matuszewski 1982). This difference in number is due to the elimination during development of chromosomes from all cells destined to become somatic cells.

There is much confusion in the literature concerning the number of chromosomes present in the germ line and somatic cells of the Cecidomyiidae. The numbers of chromosomes eliminated appear to be different for each species. Matuszewski (1982) asserts that the one constant factor regarding the cytology of the Cecidomyiidae is polyploidy of the chromosome set. Other authors such as White (1946) see the fact that the number of chromosomes in the germ line cells is often a multiple of the haploid number as being a coincidence.

Schüpbach and Camenzind (1983) and Matuszewski (1982) both say, when describing *M. speyeri,* that the oocyte nucleus contains 29 chromosomes, and that it undergoes an equational maturation division giving a primary cleavage nucleus and a polar body which degenerates. The former then undergoes 3 normal mitoses. Subsequently, in the 4th cleavage division in 7 of the 8 mitoses, 23 daughter chromosomes (E chromosomes) are eliminated from each daughter nucleus leaving these somatic cells with only 6 chromosomes (S chromosomes). Six is considered by these authors to be the diploid number for the somatic cells. The one nucleus of the eight which does not undergo chromosome elimination becomes the primordial germ-line nucleus. This

nucleus then undergoes 2 divisions to produce the 4 pole cells. described in Section 1.5.2, which will produce the oogonia.

Kunz *et al.* (1970) describe oogenesis in the paedogenetic species *Wachtliella persicariae*. In this case the somatic cells have 8 S chromosomes, while there are 30 E chromosomes eliminated. This work concentrated on elucidating a role for these E chromosomes which would appear to have no function. Kunz *et al.* were able to distinguish the E chromosomes within the oogonia as they were uncoiled. They conclude that one important role for these E chromosomes may be RNA synthesis which is essential for the oogenesis.

White (1946) studied the parthenogenetic *Miastor metraloas* which has a life cycle similar to that of *H. pygmaea* and *M. speyeri*. He describes the germ-line cells as being octoploid, having 48 chromosomes, 36 of which are eliminated in the somatic cells leaving a diploid number of 12. However Matuszewski (1982) describes Kahle's (1908) study of the cytogenetics of *Miastor metraloas* in which the germ-line chromosome number is described as 22 while the somatic cells are thought to have 11.

Matuszewski (1982) gives the chromosome number in *H. pygmaea* as 66, with the somatic cells having 11 chromosomes each after elimination.

Bantock (1970) describes chromosome elimination in *Mayetoila destructor*, a non-paedogenetic species. This species is described as having 40 chromosomes per nucleus in the pole cells. The cells destined to be somatic cells lose 32 of these chromosomes

leaving 8, while the other cells form the primordial germ cells. Bantock concludes that the genes necessary for gametogenesis have accumulated in particular chromosomes which are eliminated from the somatic nuclei, but does not offer a reason for this phenomenon.

Following elimination of the E chromosomes the new nuclear envelope formed at the end of telophase surrounds the S chromosomes but not the E chromosomes. At a later stage they become pycnotic, and then vacuolised, before finally disintegrating.

Matuszewski (1982) describes two different types of chromosome elimination: one type demonstrated by *H. pygmaea* in which the chromosomes destined for elimination do not move at anaphase, and another type, seen in *M. speyeri,* in which incomplete separation of the sister chromatids leaves the E chromosomes separated from the S chromosomes.

In the case of paedogenesis the result of this is that all of the germ cells produced are genetically identical. It is not clear however that after division and elimination of certain chromosomes whether the somatic cells are all identical or whether different sets of chromosomes are retained. If they are not always the same it may provide the Cecidomyiidae with a method of asexual clonal reproduction while still enabling them to maintain variation with different chromosomes present in the somatic cells (Panelius 1967). Such variation could occur between individuals and also between the clonal descendants of individual early embryonic cells, effectively making the larvae a somatic chimera. It should be noted, however, that chromosome elimination also occurs in non-

paedogenetic cecids (Bantock 1970) so that we cannot attribute the phenomenon to the paedogenetic way of life.

1.6 Spermatogenesis.

Spermatogenesis in male individuals of bisexually reproducing species of cecid is described by Matuszewski (1982) as being highly modified with an absence of chromosome pairing, the presence of a unipolar spindle during the first meiotic division and the production of only two sperms from each spermatocyte. The spermatocytes possess a full complement of chromosomes but the sperm that they produce carry a haploid number.

1.7 Life cycles.

Discussion in the literature about the life cycles of populations of Cecidomyiidae is confusing and often contradictory. Many different life cycles have been described (e.g. White 1946, Camenzind 1962, Went and Camenzind 1980 and Matuszewski 1982).

The accounts of the life cycles of the many species of Cecidomyiidae described by the above authors and many others show that within this Dipteran family there exists paedogenetic reproduction and parthogenetic development of eggs produced by adult flies, as well as the capacity for sexual reproduction by adult flies. The variation in methods of reproduction not only exists between species but can also be found within the same species under different circumstances.

Extensive studies of the culture of these insects and their eggs have made it possible for their life cycles to be outlined in full. Chapter 2 describes these studies in detail and will describe the work I have undertaken at Bath to elucidate the life cycles of two of the pest species of Cecidomyiidae described in Section 1.4: *M. speyeri* and *H. pygmaea.*

1.8 Environmental control of life cycles.

Matuszewski (1982) lists the many authors who have linked changes in the developmental pathways and sex determination of cecids, to many different environmental conditions. These conditions include flood, drought, light intensity, changes in nutrition and many others. A summary of the literature concerning this topic can be found in Chapter 3. Understanding environmental influences on reproduction and development, and the ability to manipulate cultures to produce males or to trigger a developmental switch, is very important in the study of the Cecidomyiidae as environmental conditions regulate the rate of reproduction and can cause populations to achieve pest status.

Chapter 3 also includes the results of experimental work carried out to manipulate laboratory populations of *M. speyeri* and *H. pygmaea* by imposing different environmental conditions.

1.9 Hormonal control of paedogenesis.

Section 1.5 described the phenomenon of paedogenesis. A hypothesis that underpinned the present research project was that the physiology of this form of reproduction must

be dependant on hormonal triggers in the same way as the normal sexual reproduction of other insect species.

Which hormones might be responsible for the control of paedogenesis in cecids? In other insects, the sesquiterpenoid juvenile hormones (JHs) and the ecdysteroid moulting hormones are both known to play important roles in the endocrine regulation of reproduction. Could it be that elevated JH levels result in the maintenance of larval characteristics when the reproductive system has matured? Alternatively, could depressed JH levels cause the insect precociously to develop the adult characteristics necessary for reproduction? The answers to these questions are very important as they may provide clues as to whether the Cecidomyiidae are suitable targets for control using Insect Growth Regulators (IGRs) such as the pesticides methoprene and fenoxycarb.

Alternatively, if ecdysteroids are involved not only in larval moulting, but simultaneously in the precocious maturation of the oocytes and the initiation of embryogenesis, then the control with ecdysteroid-agonist IGRs might be possible.

In Chapter 4 the results of experiments to discover the effect of JH and ecdysteroid analogues on the reproduction and development of *M. speyeri* are discussed, and work done to discover the identity of the ecdysteroid hormone used by *H. pygmaea* is described.

1.10 Larval nutrition.

The period of embryonic development described in 1.5.6 is associated with significant growth of the embryo. In the case of *H. pygmaea* this increase in size is about 200 fold (Ivanova-Kasas 1965). This order of size increase cannot be supported by the food reserves in the egg yolk and the growing embryo relies on the mother-larva for nutrition.

The nutritional state of the mother at this time is very important, Ulrich (1934) found a direct correlation between the size of the mother-larva and the number of offspring produced. It is true to say that not all oocytes which are produced go on to the stage of embryogenesis; some of the oocytes will degenerate and will be reabsorbed. The numbers of oocytes that do not finish development is directly dependent on the level of maternal nutrition (Matuszewski 1982).

Chapter 5 describes the production of protein by the mother larva for the nutrition of the developing embryos, and describes an ultrastructural study of the fat body of *M. speyeri*. This was undertaken to monitor changes in the maternal tissues throughout the course of embryonic development, and to shed light on the method of nutrient transfer from mother to daughter.

Chapter 2

Observations on *Mycophila speyeri* and *Heteropeza pygmaea.*

2.1 Introduction.

2.1.1 Life cycles.

Discussion in the literature about the life cycles of populations of Cecidomyiidae is confusing and often contradictory. Here the main points of the literature describing the life cycles of *M. speyeri* and *H. pygmaea* will be critically discussed.

2.1.2 *Mycophila speyeri.*

Camenzind (1962) states that adult males are very rarely seen in *M. speyeri* and that egg production by female adults is limited. He had thought that bisexual reproduction had been lost and that paedogenesis had become the sole method of reproduction in these insects. He then describes how in a personal communication from Ulrich he heard of one case in which a male and female adult had been brought to copulation and had produced eggs which had produced larvae. It is not clear whether these larvae were subsequently able to reproduce paedogenetically.

The literature is unclear as to whether when imago eggs are laid they can develop parthogenetically or whether they need to be fertilised. Matuszewski (1982) states that no parthogenetic development of adult eggs in *M. speyeri* has been seen.

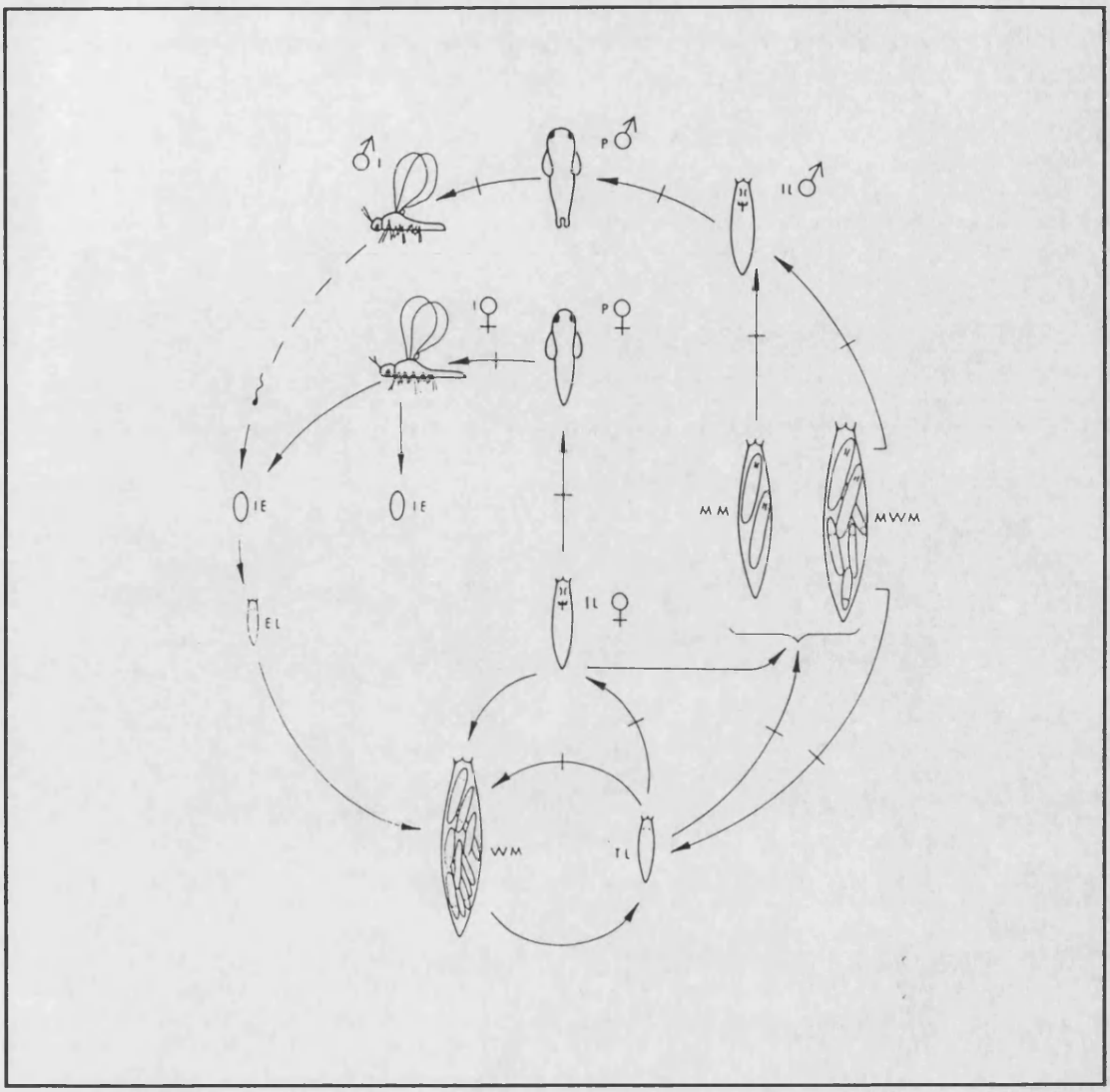


Figure 2.1 Life Cycle of *Mycophila speyeri.*

TL: female daughter larva, WM: female-mother, MM: male-mother, MWM: male-female-mother, IL: imago-larva, P: pupa, I: adult insect, IE: egg produced by an adult female, EL: fertilised egg larva, pEL: parthogenetically derived egg. (From Matuszewski 1982).
Bars across arrows indicates moults.

Unfortunately it is not clear from the literature how firmly based are these observations of adult reproduction in *M. speyeri*. In what follows the descriptions given in the literature are taken at face value. Figure 2.1 shows the typical life cycle of *M. speyeri* as described by White (1946) and Matuszewski (1982). It includes thelytokous, asexual, paedogenetic reproduction when conditions are favourable and an adult sexual cycle.

During the paedogenetic stage so called female-mother larvae give rise to daughters who will produce further generations in the same way. The literature often describes paedogenic offspring as male-mothers or female-mothers depending on the sex of the offspring they produce (Camenzind 1962, Went 1975). It should be realised that these terms do not refer to the sex of the mother larva, but to that of the offspring.

During the adult stage there is the production of sexual male and female adults that, when conditions deteriorate, migrate, lay fertilised eggs which hatch and produce paedogenetic larvae that continue the asexual cycle in the new environment.

Camenzind (1962) says that when a female larvae of *M. speyeri* develops a breastplate and widely spaced eyes and becomes destined to pupate it can, at any time, revert and become a paedogenetic mother if the conditions improved.

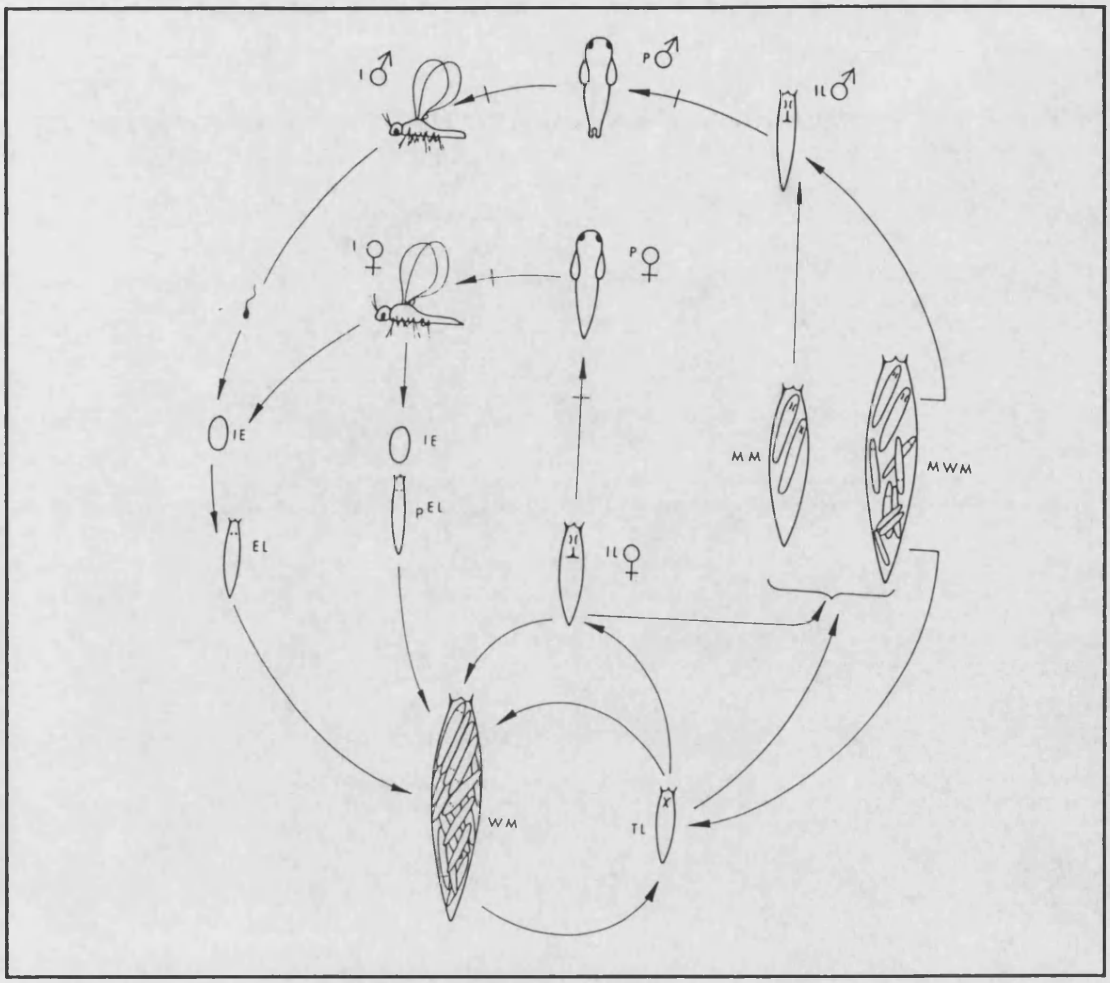


Figure 2.2 Life Cycle of *Heteropeza pygmaea*.

TL: female daughter larva, WM: female-mother, MM: male-mother, MWM: male-female-mother, IL: imago-larva, P: pupa, I: adult insect, IE: egg produced by an adult female, EL: fertilised egg larva, pEL: parthogenetically derived egg. (From Matuszewski 1982).
Bars across arrows indicates moults.

2.1.3 *Heteropeza pygmaea.*

Went and Camenzind (1980) and Matuszewski (1982) say that in their populations of *H. pygmaea* reproduction can occur not only in the larval form by paedogenesis but can sometimes occur also in the adult stage (Figure 2.2). These authors describe how, as adults, *H. pygmaea* lays eggs which are always female determined. These eggs are apparently able to develop whether they are fertilised or unfertilised. Camenzind (1962) found that equal numbers of fertilised and non fertilised eggs hatch in *H. pygmaea*. The same number of eggs are laid by the adult females whether they are virgins or not (Matuszewski 1982).

In these studies the offspring which are produced by *H. pygmaea* during paedogenesis could be male- or female-determined. Went and Camenzind (1980) state how the development time for male- and female-determined eggs differed and that they could be distinguished morphologically.

Went (1975) says that determining environmental conditions decide whether the larval ovaries in *H. pygmaea* will produce male- or female-determined eggs. The effect of environmental conditions on the sex of offspring and developmental fate will be discussed in Chapter 3.

Went and Camenzind (1980) describe how the unusual form of meiosis, described in Chapter 1, was different for the three different types of eggs produced by *H. pygmaea*, male and female determined and imago. In the imago and the male-determined eggs reduction division of the 66 chromosomes was followed by elimination of

chromosomes in the somatic cells resulting in a somatic chromosome numbers of 5. In the female-determined eggs there was a maturation division and chromosome reduction in early cleavage leaving a somatic chromosome number of 10. These observations support the proposal that sex determination in the Cecidomyiidae is the result of a haploid/diploid mechanism as in the Hymenoptera.

Wyatt (1961) documents an account of pupal paedogenesis in *H. pygmaea*; this is the only account of paedogenesis taking place in any stage other than the larval instars. Matuszewski (1982) claims that even if the offspring emerge from the pupal cuticle, egg development would still have taken place during the larval stage and claims that there is no difference between larval and pupal paedogenesis. However, the matter has not been examined in detail. It should be pointed out that egg development might well have taken place during the pharate pupal period, after apolysis.

2.1.4 Other species.

Wyatt (1961) claims that there is adult reproduction and that this occurs parthogenetically. He describes how almost all the adults produced in his culture of an unspecified cecid species were female, that they laid around 4 eggs each which developed parthogenetically and hatched into paedogenetic individuals which continued the paedogenetic cycle. He raises doubts as to whether bisexual reproduction can occur.

Published information on other species is very limited. The review by Matuszewski (1982) summarises the work carried out by other authors on the life cycles and

cytology of different species of cecid including: *Tekomyia populi, Mycophila nikoleii, Mycophila barnesi* and *Henria psalliotae.*

2.1.5 Descriptions of different sexes.

The literature refers to both male and female larvae of the Cecidomyiidae. The ability to distinguish between the two appears to be essential in order to fully understand their life cycles. Descriptions of the differences between the two sexes are few and are often confusing.

Harris (1924) describes the debate as to whether male larvae exist in paedogenetic cecids as "speculative and often in the absence of suitable data". Camenzind (1962) describes male larvae of *M. speyeri* as being significantly larger than their female counterparts and says that they can be identified because they have eyes which are very widely spaced. They then produce a breast plate or spatula sternalis and pupate to produce male imagoes. He then describes the females as also having clearly separated eyes and a breast plate.

Larvae of the many species of Cecidomyiidae are known to have a structure known as the spatula sternalis or breast plate. It is not seen in paedogenetically reproducing larval populations but is observed only in larvae destined to pupate. There have been many proposed functions for this structure; a pseudopod, a scraper or digger, or a structure used in locomotion (Harris 1923). As it is only seen in those insects which are destined to pupate it is most likely that this structure is used in some activity

peculiar to the pupal or adult stage, perhaps in moulting or in fixing the pupa to the substrate.

Harris (1924) says that among previous authors no males had been reported as arising from larvae known to have been produced by paedogenesis. He states that in a culture of *Oligarces* sp. adults produced were in the ratio 1:1 male to female. He differentiated between adult males and females by the identification of sperm on the dissection of the gonads.

Harris (1924) describes how cecid males and females are never produced by the same individual and that male- and female-producing individuals are indistinguishable from one another. Matuszewski (1982) also refers to male *H. pygmaea* larvae as being substantially larger than the females with widely spaced eyes.

White (1946) describes two different types of larvae in *Miastor metraloas*: one large type with widely separated eye spots and one which develops a sternal spatula in the final instar. Rather than being descriptions of male or female larvae he describes them both as being sexual types which go on to produce adults rather than continue with paedogenesis.

2.1.6 Culture techniques.

Culture techniques for different species of Cecids are described in a number of articles in the literature (Harris 1923, 1924, 1925, Camenzind 1962). Except for Harris (1923) all describe a similar culture technique using malt-extract agar plates, inoculated with a

fungal culture, incubated at around 20 - 25 °C. The culture method used by Harris (1923) was not sophisticated and involved keeping the cecids on the wood on which he had found them in the field in a tin box.

Harris (1925) did not inoculate the plates with a single species of fungus but allowed the larvae to inoculate the plates with the micro-organisms that they had on their cuticle. Wyatt (1961) either cultured his cecids on *A. bisporous* cultured on malt-agar slopes or on *A. bisporous* cultured on commercial mushroom compost in Kilner jars. In the earlier literature (Camenzind 1962) the fungal species used to grow the cecids on was *Peniophora albula* (now known as *Corticium evolvens*). Later papers, such as Went and Camenzind (1980) use *Chondrostereum purpureum* or *Stereum hirsutum.*

2.1.7 History of *in vitro* culture.

Went (1971) describes a technique that he used for the culture of *H. pygmaea* eggs *in vitro*. He used this technique in order to monitor the development of the eggs and embryos from when they are released into the haemocoel of the mother larvae to maturation. As there had not been any *in vitro* culture work done on these insects previous to this paper he was unable to use an established defined culture medium. Instead he used the haemolymph from sterile donor larvae as the medium for growth of the embryos.

Mother larvae containing developing embryos were exposed to X rays. These offspring developed normally and emerged but did not produce offspring of their own. When they had grown to a maximum size they were dissected and eggs from a viable larva

were added to the haemolymph. Donor larvae of this size were used because they contained a maximum amount of haemolymph and their fat bodies had dissociated into separate cells. The fat bodies from the egg-donating larvae were removed and the eggs were left to grow suspended in a drop of the haemolymph from an inverted coverslip sealed onto a cavity slide.

Went (1978a) observed that using this technique growth and development followed a similar path and time course to growth *in vivo*. He found that the presence of dissociated but intact fat body cells was essential. They provided nutrients for the developing embryos; he found that if the cells were damaged in any way or if the fat body lobes had not dissociated into separate cells then the embryos did not thrive.

In subsequent papers Went (1978b) was unable to get the *in vitro* culture technique to support the development in its entirety and of the eggs cultured most did not go on to produce fully grown larvae. The role of the fat body in the development of embryos will be discussed further in Chapter 5.

2.2 Materials and Methods.

2.2.1 Culture of *Mycophila speyeri* and *Heteropeza pygmae.*

There are four cecid cultures at Bath: three lines of *M. speyeri* and one of *H. pygmaea.* Two of these cultures of *M. speyeri* originated from the laboratory of Dr. P. White, (H.R.I., Wellesbourne) and one is a culture isolated from an infestation at a local mushroom farm. The culture of *H. pygmaea* was also supplied by Dr. P. White.

The insects were cultured according to a method supplied by Dr. P. White. To maintain a paedogenenetic culture for experimentation the larvae were cultured on 9 cm Petri dishes (Sterilin), each containing 40 cm^3 of 2% malt extract (Lab M), agar (Oxoid). Each dish was inoculated with a culture of *C. purpureum* three days prior to inoculation with the larvae.

Before being placed onto the plates using a 00 sable brush dipped in 70% denatured alcohol each insect was dipped briefly into 2% formaldehyde and then rinsed in sterile distilled water before being dried on piece of tissue and placed on the dish. The plates were kept in the dark in a temperature-controlled incubator (Gallenkamp) at 25°C.

These cultures were prepared twice weekly in order to provide a constant supply of paedogenetic larvae for experiments. Some cultures of the above were maintained in the fridge at 4°C on cultures of *S. hirsutum* on malt-agar slopes. This was in order to maintain stock cultures for long-term storage.

Culture was attempted on plates containing the commercial fungus *Agaricus bisporous* in order to replicate conditions found on mushroom farms. When this fungus was cultured in the laboratory it was not possible to create optimal conditions for the fungus to grow quickly enough to inoculate the plates with insects.

Any adult flies produced on the routine cultures were sexed by examination of the shape of the abdomen and by looking for the production of eggs on dissection.

The two species of fungus used to feed the insects, *C. purpureum* (isolates UIIFB and KII) and *S. hirsutum* (isolates Homokaryon 8 and Heterokaryon W), are two species of wood-rotting fungus, both non sporulating basidiomycetes. The fungal cultures were supplied by Dr. A. D. M. Rayner (University of Bath).

2.2.2 Fixing and sectioning for light microscopy.

Whole *M. speyeri* larvae were prefixed under vacuum for 10 minutes and then overnight in 2.5% glutaraldehyde (Agar Scientific Ltd., Stansted, U.K.) and 7% acrolein (Agar Scientific Ltd.) in a washing buffer; 0.6% sucrose (BDH) and 0.018M phosphate buffer at pH 7.8(Appendix 1).

They were washed in 0.6% sucrose in 0.018M Sorensen's phosphate buffer at pH 7.8 before being postfixed in 1% osmium tetroxide (Agar Scientific Ltd.) in buffer for one hour. Following the postfix stage they were washed in distilled water before being dehydrated in an acetone series.

The samples were imbedded in TAAB premix resin (TAAB Laboratory, Aldermaston, U.K.) which was polymerised at 24 °C for 24 hours and then sectioned using a glass knife on a Reichert OMU3 microtome (Leica U.K. Ltd, Milton Keynes, U.K.).

0.5 μm thick sections were mounted on glass slides stained using toluidine blue in 1% borax solution and mounted in DPX (BDH) and examined and photographed using an Olympus BH2 microscope.

2.2.3 Culture of ovaries, follicles and embryos.

As was described in 2.1.7 Went (1971) cultured eggs and embryos *in vitro*, using the haemolymph from X-ray-sterilised individuals as a culture medium. Ideally the paedogenetic eggs would be cultured in a chemically defined medium. If this was possible it would be simple to alter growth conditions such as the hormone concentration and to see very clearly what effect this had on development.

The insect culture medium TC-100 (Sigma) was used. Larvae of *M. speyeri* were dissected in 2μl of culture medium on a well-slide. *M. speyeri* were used rather than *H. pygmaea* because the larvae and embryos are orange in colour and are more visible than the embryos of *H. pygmaea*. The empty cuticle of the donor larva was removed leaving a drop containing: the eggs, the intact fat body, the haemolymph and the culture medium. Eggs and embryos of different ages were used.

Although in his experiment Went (1971) used the dissociated fat body cells of *H. pygmae* it was not thought that using intact lobes from *M. speyeri* would be a problem

as fat body cell dissociation never occurs in this species. A small amount of Vaseline was smeared around the well and a coverslip was placed on top in order to form a sealed chamber. Oxygen was passed over the culture drop at regular intervals and the drop was monitored carefully to check for signs of drying out. The slides were kept at 25 °C, the temperature normally used for culture. Development within the drop was monitored carefully for signs of change on an Olympus BH 2 microscope.

2.3 Results.

The culture techniques described in 2.2.1 were suitable for maintaining viable cultures of both *M. speyeri* and *H. pygmaea.* When placed on the agar plates the larvae grazed on the fungal mycelium and moved around on the plate (Figure 2.3). The white larvae of *H. pygmaea* appeared to be far less active than the orange larvae of *M. speyeri.*

The mode of reproduction seen in the cecid cultures in the laboratory was exclusively that of thelytokous paedogenesis; the asexual production of only females while still in the larval form. For as long as conditions were favourable on the agar plates all four cecid lines reproduced in this way.

During this study, all four lines underwent ca. 100 - 150 generations over a period of three years. Matuszewski (1982) noted that using the type of laboratory culture techniques that are described in 2.2.1 that he had cultured around 500 generations of *H. pygmaea* paedogenetically over a period of 8 years by ensuring that they were permanently in favourable conditions.

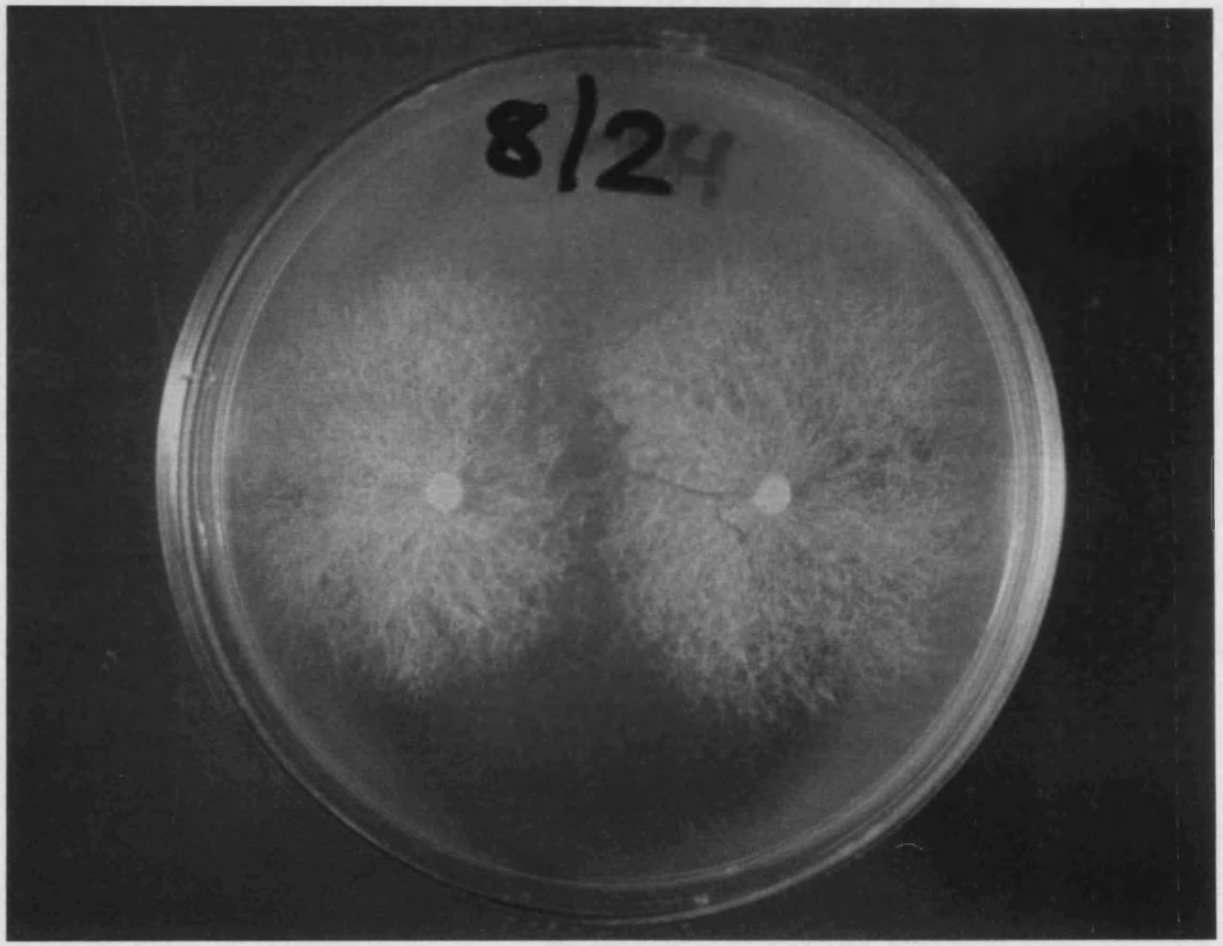


Figure 2.3 Petri dish containing 2% malt extract agar inoculated with *Chondrostereum purpureum* and *Mycophila speyeri* (× 1.5).

2.3.1 Life cycles.

Figure 2.4 shows the life cycle that was observed both for *M. speyeri* and *H. pygmaea* cultures at Bath. The cycle is a subset of the more complex cycles observed by Went and others, shown in Figures 2.1 and 2.2 (Matuszewski 1982). These are described in some detail in the next 2 sections.

2.3.2 *Mycophila speyeri.*

During routine culture in the laboratory the *M. speyeri* larvae would feed on the fungal mycelium and increase in size. The larvae of this species are orange in colour. This is due to the orange colouration of the fat body (Figures 2.5 and 2.6). During the first three days of growth this fat body increased in size as the larva fed. After 3 days paedogenetic embryos could be seen developing in the haemocoel of the mother-larvae (Figure 2.7).

During the 4 - 5 day stage these embryos developed into larvae, firstly becoming orange in colour as their fat bodies developed, and developing eye spots. Finally during the fifth day they began to move independently within the maternal cuticle. During the fifth day they emerged through a tear in the maternal cuticle which normally developed around either the mouth or the anus. After the emergence of the offspring all that was left of the mother-larva was the empty cuticle and some tracheae (Figure 2.8).

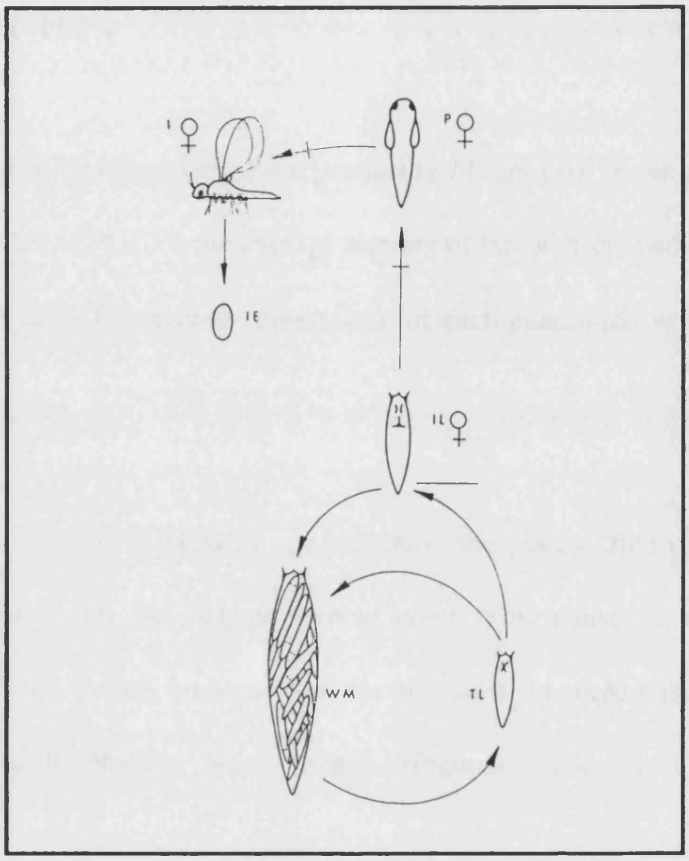


Figure 2.3 Diagramatic representation of the life cycle of both *M. speyeri* and *H. pygmaea* at Bath.

TL: female daughter larva, WM: female-mother, IL: imago-larva, P: pupa, I: adult insect, IE: egg produced by an adult female. (After Matuszewski 1982).
Bars across arrows indicates moults.

These offspring larvae moved away from the empty maternal cuticle and immediately began to feed. If conditions remained favourable they produced another generation themselves within 5 days.

From a sample of 50 paedogenetically reproducing *M. speyeri* larvae cultured under the conditions described in 2.2.1 the average number of larvae produced per generation was 16.03 (± 7.43 SD). The average time taken for each generation was 5.24 days (± 0.91 SD).

When these cultures were allowed to age naturally, the plates dried out, the insects became overcrowded and the fungus became older. This caused a developmental switch to occur in the insects on some but not all plates. In these cases pupae were produced and adult insects were found (Figures 2.10, 2.11 and 2.12).

Figure 2.5 Day 1

Figure 2.6 Day 3

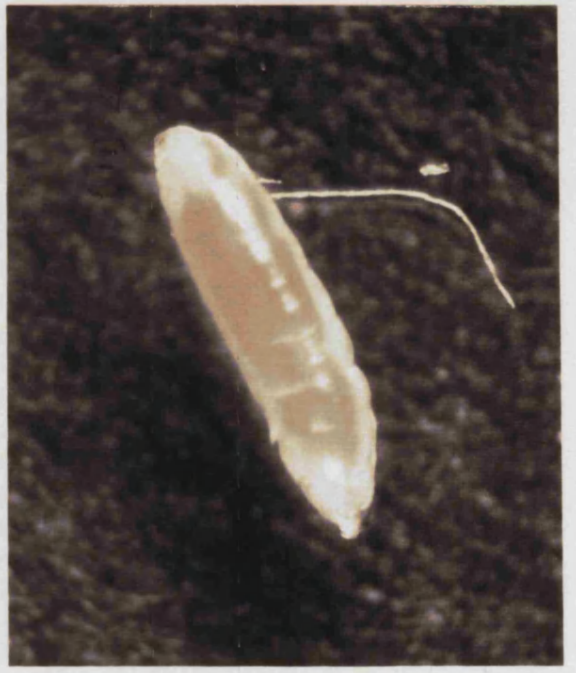


Figure 2.7 Day 5

Paedogenetic *Mycophila speyeri* larvae (×20)

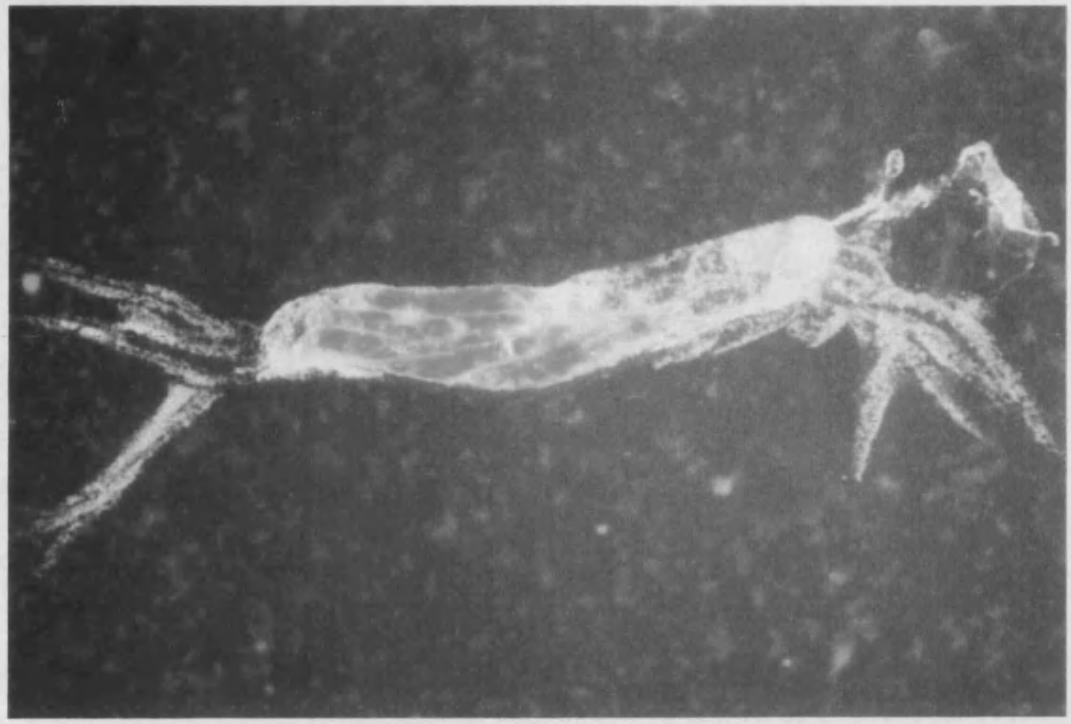


Figure 2.8 Five day old, paedogenetic, *Mycophila speyeri* 'mother larva' with offspring emerging (× 20).

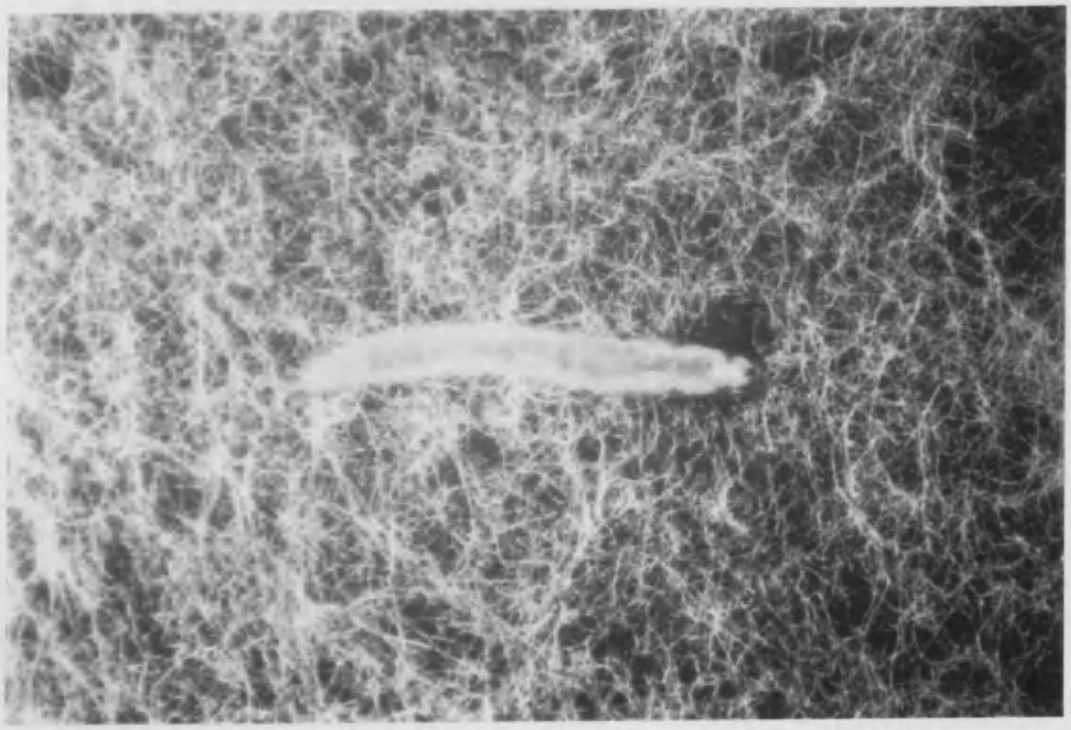


Figure 2.9 Three day old, paedogenetic, *Heteropeza pygmaea* larva (×20).

Figure 2.10 Pre-pupal larva.

Figure 2.11 Pupa.

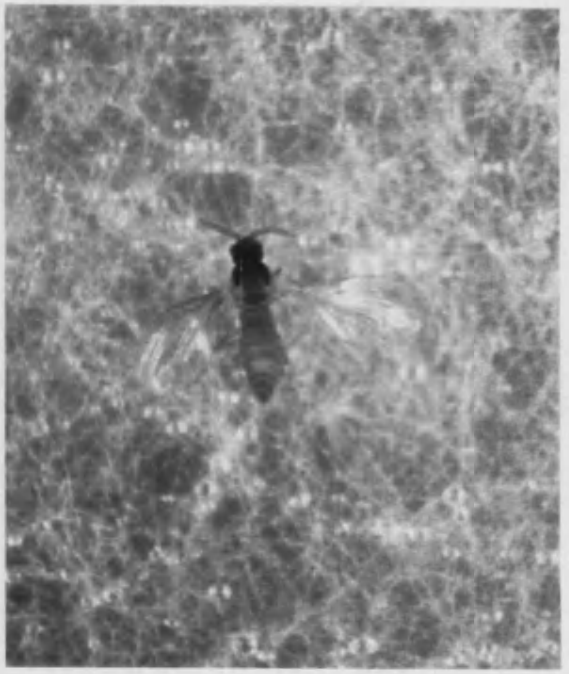


Figure 2.12 Adult fly (female).

Mycophila speyeri from the adult cycle (×20).

At no time during routine culture did a larvae showing pupal characteristics (spatula etc.) revert to the paedogenetic form when transferred to optimum conditions in contrast to the descriptions of Harris (1925) and Matuszewski (1982).

All adult flies produced during routine culture were examined closely in order to determine whether they were male or female. From a sample of 100 adults examined all appeared to be female, clearly having an ovipositor and containing eggs on dissection. At no point during routine culture were any males produced.

Although the adult female flies produced eggs, some of which were laid, none of the eggs examined hatched so that no paedogenetic generations arose from adult insects during this study. This was not the finding of Matuszewski (1982) (compare Figure 2.3 with Figure 2.1).

2.3.3 *Heteropeza pygmaea*.

The culture of *H. pygmaea* larvae (Figure 2.9) behaved in a very similar way to that of *M. speyeri*. While conditions remained favourable the larvae maintained a paedogenetic cycle. The average time taken for each generation to be produced was slightly longer at 6.7 days (± 0.67 SD) and produced more insects per generation 19.54 (± 5.89 SD).

When adult flies were produced no males were seen and although eggs were laid by the adult females they did not hatch and develop into paedogenetic larvae, unlike the life cycle seen in Figure 2.2.

Unlike the larvae of *M. speyeri*, when the newly emerged offspring of *H. pygmaea* left the maternal cuticle they did not move away. Instead they remained around the dead mother larvae where they reproduced. This resulted in dense masses of larvae on the agar plate which makes this species very easy to collect for experimentation.

Occasionally in the cultures of *H. pygmaea* kept in the laboratory examples of what Wyatt (1961) describes as 'hemi-pupae' have been seen. In this case the cuticle appears to be desiccated and brown in colour and the individual enters a diapause phase. This has often been seen in cecids collected in the field. If the 'hemi-pupa' is dissected it is found to contain one or two paedogenetic larvae. If this 'hemi-pupa' is moved to favourable conditions then these offspring emerge. Although this is described as a separate phenomena by Wyatt, he raises doubts as to whether this is reproduction occurring at the pupal stage or whether the 'hemi-pupa' is a resistant stage during paedogenetic reproduction. This has not been observed in *M.speyeri*.

It should be noted that there is much natural variation in the number of larvae produced per generation by both *M. speyeri* and *H. pygmaea* larvae. This has made it difficult in subsequent experiments to see the effects of various treatments when the background fluctuations are so high. This phenomenon has also been noted by Wyatt (1961).

When culture plates of both species were seen to deteriorate, becoming overcrowded and producing pupae and adults, larvae were often seen migrating and moving into the

drops of condensation which form on the lids of the Petri dishes where they would become trapped.

The diagrams showing the life cycles of *M. speyeri* and *H. pygmaea* (Figures 2.1 - 2.3) indicate that these insects moult at various stages in their life cycle. This is undoubtedly the case. It was difficult to pinpoint the times of these moults as the discarded cuticle was almost invisible and newly moulted insects were no different in appearance to those about to moult.

2.3.2 Follicle and ovarian development.

To observe internal structures, it was necessary to fix and embed the developing cecid larvae. The fixation of entire larvae proved to be very difficult. Even if the larval cuticle was pierced to allow penetration of the fixative the embryos inside the mother larvae were very resistant and often failed to be well fixed and preserved. While this meant that the observation of the early stages of development was difficult the latter stages, from the formation of the blastocyst onward, could be seen.

In all of the stages of paedogenetic larvae examined the following features were clearly visible: the fat body, the gut, the cuticle and the embryos (Figure 2.13). Figures 2.13 and 2.14 show longitudinal cross sections through two 3 day old *M. speyeri* larvae. The fat body cells can be seen to lie in ribbons within the haemolymph from the head to the anus of the larva. The gut can clearly be seen in both sections as can the paedogenetically developing embryos which are dispersed within the haemolymph. The

creeping welts found on the ventral side of the larva which aid locomotion can be seen in Figure 2.13 as can the muscle cells which lie beneath the cuticle.

One interesting phenomenon was seen in the day 4 and 5 preparations. This is shown in figures 2.13 - 2.19; the embryos appear to be in direct contact with the fat body cells. This confirms the findings of Ivanova-Kasas (1965) who mentioned 'the direct contact between the trophocytes and the cytoplasm of the ovocyte' and linked the association of the fat body cells and the developing embryos during this unusual form of reproduction with the question of the larval nutrition.

Figure 2.18 shows an embryo which had only been released into the maternal haemolymph from the ovary for 24 hours. It had reached the blastoderm stage. The follicular epithelium which surrounds the developing embryos in this species can clearly be seen.

Figure 2.15 shows a transverse section through a 2 day old larva. The tissue within this insect is poorly fixed and detail is difficult to distinguish. The dominant feature within this insect is the gut and the fat body. Figures 2.16 and 2.17 show transverse sections through 4 and 5 day old *M. speyeri* larvae. In both sections the gut and the developing embryos can be seen. The major difference between the two sections is the size of the embryos that they contain and the appearance of the fat body cells. The fat body cells of the 5 day old larva, which can be seen at greater magnification in Figure 2.19, appear to be less densely stained. This may be due to the fact that they contain less

nutrient reserves than those in the 4 day old larva and that these nutrients are used in the nutrition of the developing embryos. This will be discussed at length in Chapter 5.

2.3.3 *In vitro* culture of ovaries, follicles and embryos.

Ovaries, follicles and embryos were removed from the larvae of *M. speyeri* as described by Went (1971) in Section 2.1.8 and cultured along with the haemolymph and intact lobes of the fat body as described in Section 2.2.3.

The eggs and the embryos appeared to remain alive for a number of days but did not show any signs of development. The majority of cultures remained free of microbial growth so that the failure of the eggs and embryos to develop was not a consequence of contamination. Although no development of the ovaries and follicles took place during this experiment the close study of ovaries and follicles dissected from the mother larva gave the opportunity to identify certain features:

The follicular epithelium could be seen around the eggs and embryos of all stages. It can be seen around the egg follicles in Figure 2.20 and around the fully developed embryo shown in Figure 2.25. In Figure 2.25 it can be seen that it completely surrounds the embryo, preventing direct contact between the embryo and the maternal tissues.

Figure 2.20 shows the egg follicles dissected from a newly emerged *M. speyeri* larva. The egg follicles in this species are retained within the ovary until 24 hours after the mother larva has herself emerged and begun to live freely.

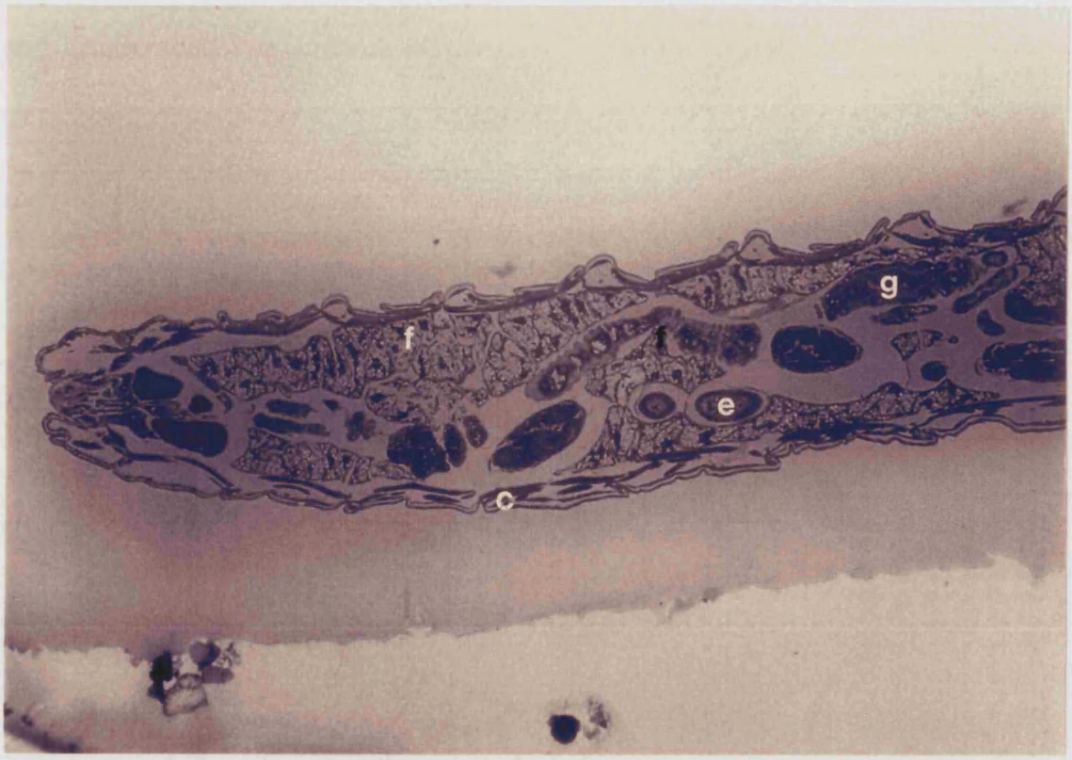


Figure 2.13 Longitudinal section through 3 day old *Mycophila speyeri* larva (× 250)

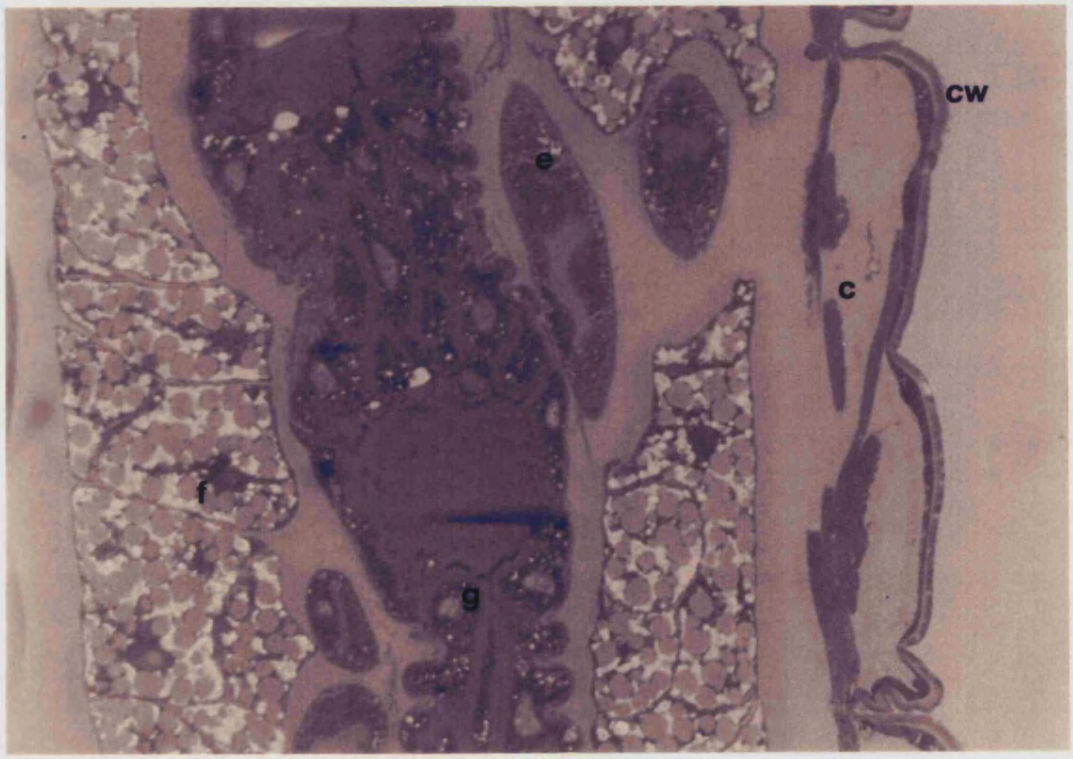


Figure 2.14 Longitudinal section through 3 day old *Mycophila speyeri* larva (× 450)

(Key f = fat body, e = embryo, g = gut, c = cuticle, cw = creeping welts).

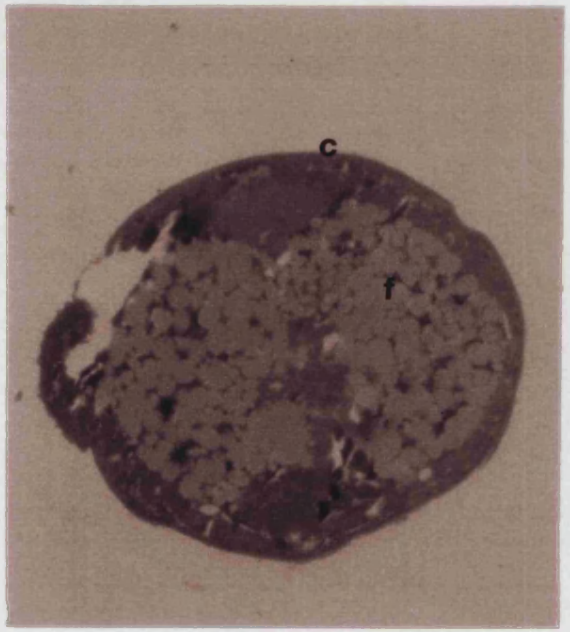


Figure 2. 15 Day 2 larva

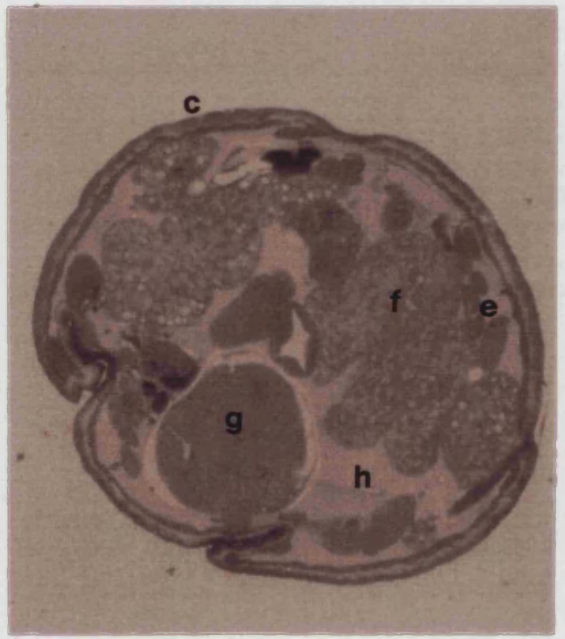


Figure 2.16 Day 4 larva

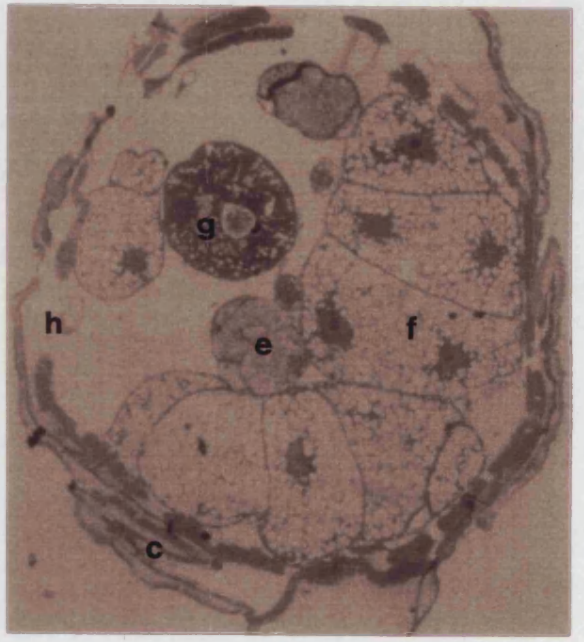


Figure 2.17 Day 5 larva

Transverse sections through paedogenetic *Mycophila speyeri* larvae (× 100)

(Key f = fat body, e = embryo, g = gut, c = cuticle, h = haemolymph)

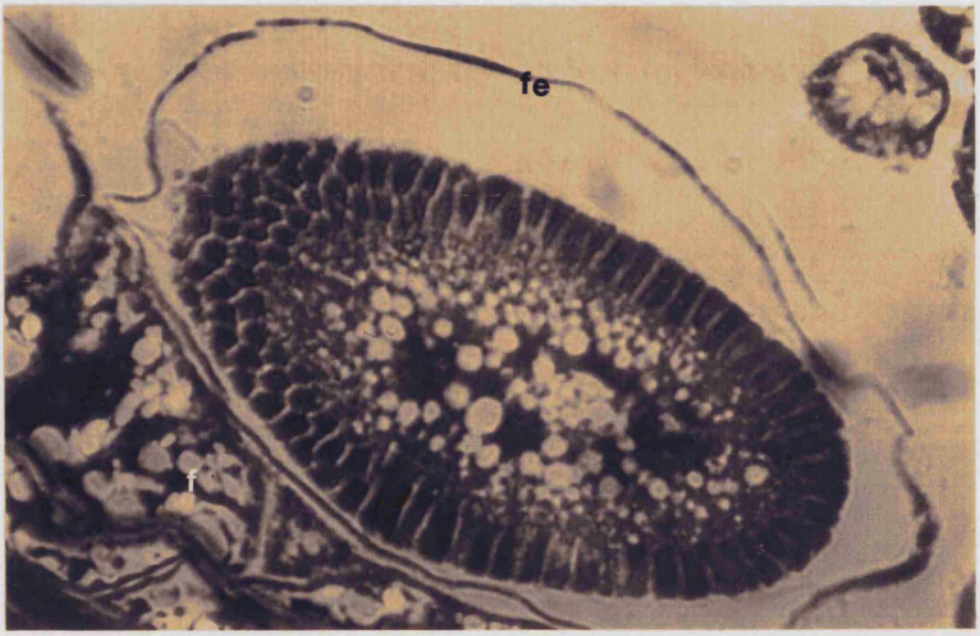


Figure 2.18 *Mycophila speyeri* embryo at 24 hours (× 1000)

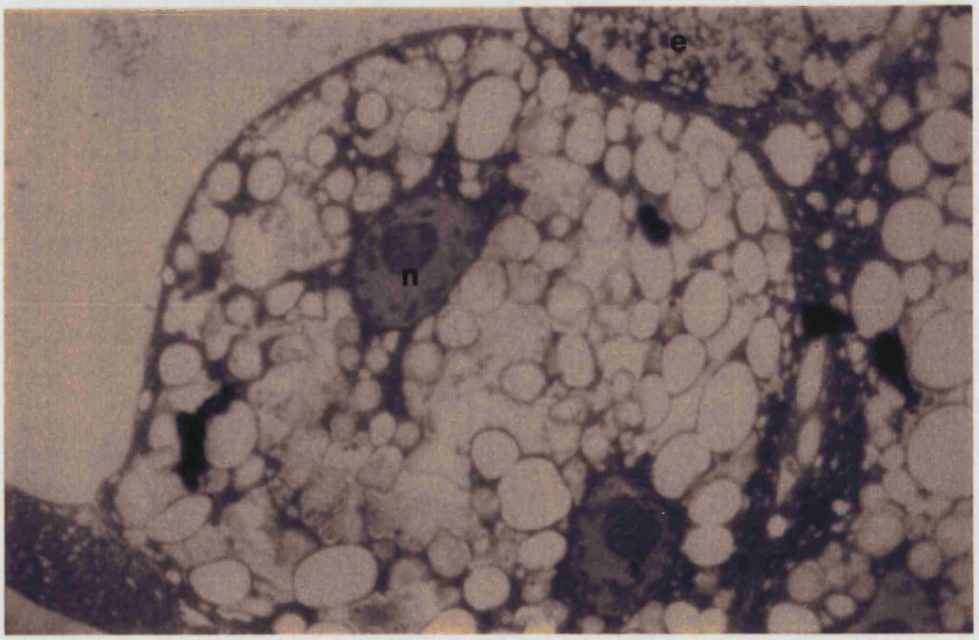


Figure 2.19 Fat body cells from a day 5, paedogenetic *M. speyeri* larva (× 1000)

(Key f = fat body, fe = follicular epithelium, e = embryo, n = nucleus)

After 24 hours of being contained within the ovary the oocytes, as described by Matuszewski (1968) and Schüpbach and Camenzind (1983) are released into the maternal haemocoel. This was observed in the *M. speyeri* culture maintained at Bath, where any paedogenetic larvae which were older than 24 hours were found to contain individual egg follicles (Figure 2.21) in dissection. Figure 2.20 shows follicles which have not been released into the haemocoel, the nurse chamber and the oocyte can be seen.

Figures 2.22 and 2.24 show embryos removed from 2 and 3 day old larvae respectively. The embryo from the 3 day old larva is at the germ-band elongation stage. These embryos did not develop further after they had been removed from the mother larvae. Figure 2.23 shows the same embryos seen in Figure 2.22 after 24 hours in *in vitro* culture, some degeneration had occurred.

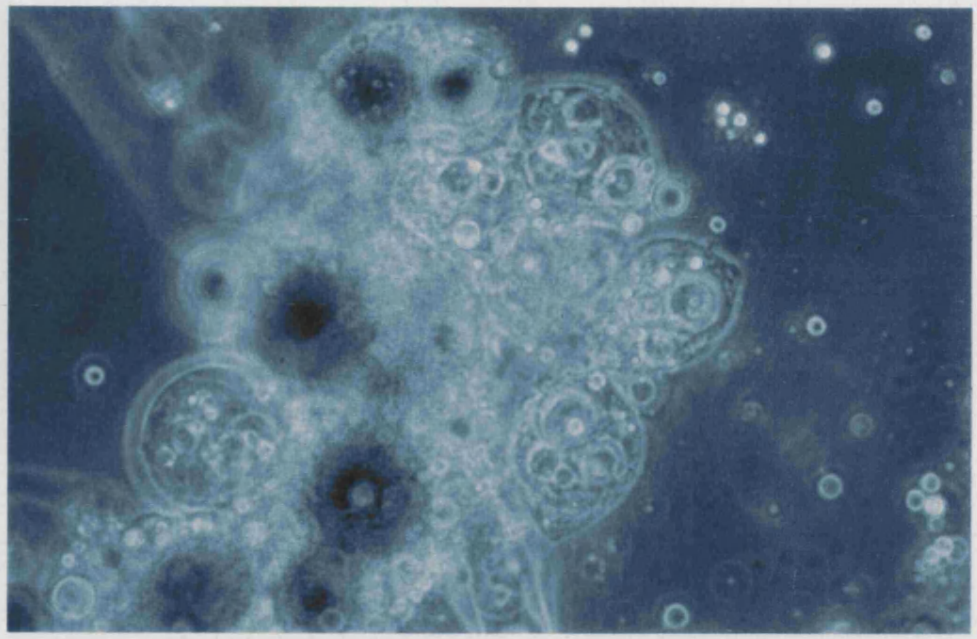


Figure 2.20 Egg follicles in the ovary of a newly emerged *M. speyeri* larva (× 1000)

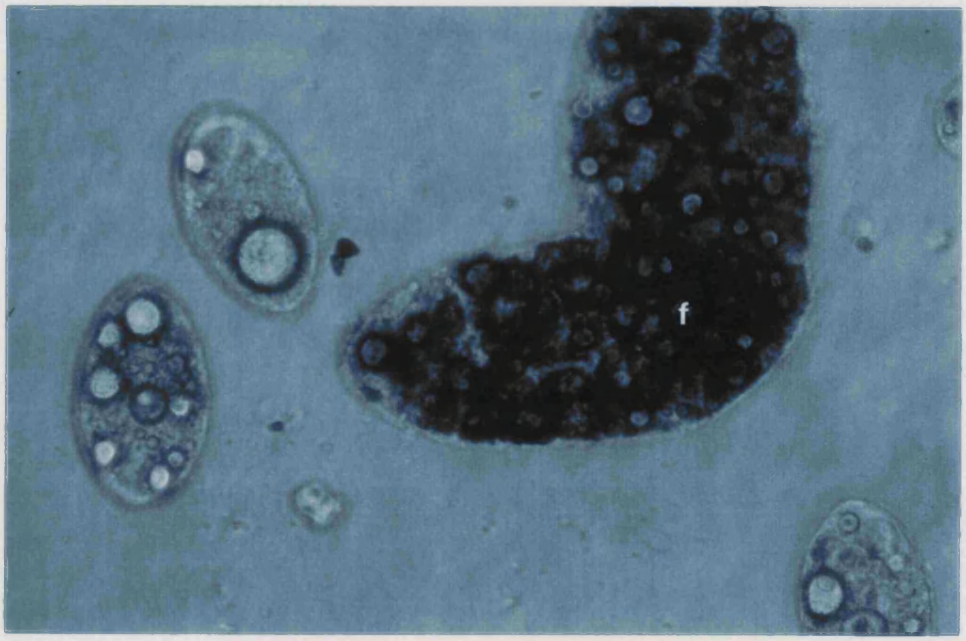


Figure 2.21 Individual egg follicles from 24 hour old *M. speyeri* larva (× 1000)

(Key f = fat body)

Figure 2.22 Embryos from a 2 day old *Mycophila speyeri* larva (× 100)

Figure 2.23 Embryos from a 2 day old *Mycophila speyeri* larva, after 24 hours in culture (× 100)

Figure 2.24 Embryos from a 3 day old *Mycophila speyeri* larva (×100)

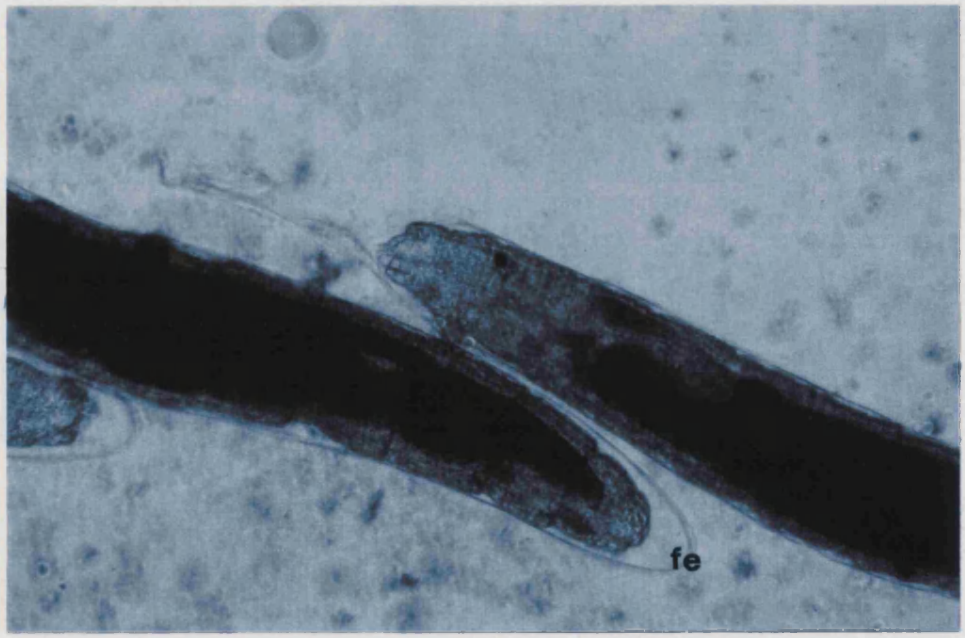


Figure 2.25 Fully developed *Mycophila speyeri* embryo from a 5 day old 'mother larva' (× 100).

(Key fe = follicular epithelium)

2.4 Discussion.

Four lines of Cecidomyiidae were cultured in the laboratory on the fungus *C. purpureum* using the method described in 2.2.1. The three lines of *M. speyeri* and the one line of *H. pygmaea* followed the life cycle shown in Figure 2.3.

While conditions remained favourable, the plates were fresh and uncrowded and the fungal mycelium was growing; all four populations maintained a paedogenetic cycle. If the plates deteriorated, the fungal mycelium became old and dry and the plates became overcrowded; larvae were produced which had a sternal spatula which were destined to pupate. The pupae produced developed into adults. The adults produced were all female and laid eggs which failed to hatch.

It is clear that many authors document a slightly different life cycle to that seen in the four lines from two species maintained at Bath. It is apparent from a close reading of the literature that only anecdotal accounts of cecid cultures producing males which mate with female adults and give rise to paedogenetic individuals exist. It is unfortunate that a clearer indication was not given in previous papers of the frequency with which adult males were observed. It is these papers which lead to the assumption that a sexual part of the lifecycle exists for this group of insects.

Although systematic studies of large numbers of the adult flies produced from all 4 lines maintained at Bath were undertaken no males were seen. It is possible that the lines studied at Bath differed from those studied by Went and co-workers, for example, in being unable to produce males. It may be that if a line of paedogenetic cecids

maintains a paedogenetic cycle for a period of time it becomes reproductively isolated and it may be possible that the lines at Bath have lost the ability to produce males.

If no males can be produced by paedogenesis and eggs produced by female adults cannot develop parthogenetically then males cannot be produced. Alternatively, if the production of males is very rare, then insufficient observations may have been made.

From the literature it is fair to assume that there are cases in other cecid species and in particular lines of *H. pygmaea* and *M. speyeri* in which either males or parthogenetically producing adult females are found.

A further difference between my observations and previously published work concerns the plasticity of the developmental pathways. In this study it was not found to be the case that after producing what we will call a larvae destined to pupate, an individual could revert and become a paedogenetic individual, as described by Camenzind (1962). Once an individual was born with a sternal spatula and widely spaced eyes that individual invariably pupated and produced an adult. Placing that individual onto a plate with favourable conditions could not change its developmental pathway.

Camenzind (1962) describes the males as having a breastplate and pupating to produce imagos. In the culture here at Bath all individuals destined to pupate had a breastplate, yet all went on to produce female adults. In this case the description given by White (1946) accurately described the difference between paedogenetically reproducing

larvae and those individuals destined to pupate, and not between insects of different sexes.

This study shows that the conditions in which the larvae are cultured is an important factor in determining the developmental pathway that the insects follow. One or more of the conditions present in an ageing culture may be the cause of the developmental switch. Chapter 3 describes how the environmental conditions in which the cecids are cultured can be manipulated in an attempt to produce adult insects and to affect a switch in the developmental pathway.

The behaviour exhibited by these insects may be indicative of their culture in the laboratory. It may be useful to collect a number of field populations to see whether other populations can produce males or to collect and screen numbers of adults collected from the field to see whether male adults could be found and if so can they copulate with female adults to produce viable eggs.

It may be possible to culture cecid populations in conditions more similar to those found in the field, viz. on decaying wood which supported many generations, as in Harris (1923). This would reflect the limitations brought about by overcrowding, unlike the one or two generation cultures kept in the laboratory. The disadvantage of this would be that the resulting culture would be affected by a very wide range of unknown factors including those arising from the wood and fungal interactions.

Adult sexual reproduction in these insects is often seen as the dispersive stage (Harris 1924) and as such a vital part of the life cycle in order to maintain populations. Paedogenetic cecids can however be successful in the field without this stage in their development. In mushroom farms where *M. speyeri* and *H. pygmaea* can cause a large amount of economic damage the larvae are believed to spread from mushroom house to mushroom house without entering the adult stage, by sticking to the clothes of workers and moving with them from house to house (personal communication by a mushroom farm proprietor).

Certain species such as *H. pygmaea* can also produce diapause stages which consist of a viable daughter larvae inside the dried skin of the mother. It is this that Wyatt (1961) describes as pupal paedogenesis. If these two stages can facilitate dispersal then these insects may not need to rely on a sexual stage to spread.

Harris (1925) states that adult sexual reproduction is not essential to the continued existence of these species for as long as conditions suitable for paedogenetic reproduction exist. Although this does not confirm that there is no sexual reproduction it supports the findings of this study; that paedogenetic reproduction is successful and that production of males and sexual reproduction is rarely seen.

There are a number of reasons which may explain the failure of eggs from *M. speyeri* larvae to grow *in vitro* during this study. Went (1971), in his work on *H. pygmaea* eggs and embryos, used fat body cells to provide nutrition for the growing eggs. He restricted the cells used to those from older larvae which had dissociated fat body lobes

to produce single cells. This is a feature of the fat body cells of older *H. pygmaea* larvae which does not occur in *M. speyeri.* In this experiment entire fat body lobes were used. The use of non-dissociated fat body cells may have been detrimental to the eggs in this experiment.

Went (1971) describes a failure of eggs *in vitro* to develop if the fat body cells were damaged in any way during dissection due to a release of enzymes which are detrimental to the eggs. It may have been the case that the fat body cells were damaged in this experiment during the dissection of the larvae.

Went (1971) used the haemolymph from older sterilised insects to provide the culture medium for his eggs and embryos *in vitro.* It is possible that even though the haemolymph from the egg-donor larvae was included with the culture medium in this experiment that there were some missing growth factors which meant that the eggs and embryos could not develop. This is an important factor during the *in vitro* culture of eggs which have been removed from the whole insect and the endocrine glands of that insect. Although this was also the case in Went (1971) he did use haemolymph from sterilised insects as the culture medium which presumably contained these missing growth factors.

More work is needed on the culture of paedogenetic eggs *in vitro.* If these eggs could be removed from donor larvae and cultured *in vitro,* it would be an ideal opportunity for the manipulation of development. It would enable the direct use of IGRs and enable

us to learn more about a unique set of hormone conditions: larvae remaining juveniles whilst being able to produce offspring.

Chapter 3

Environmental Control of Development and Reproduction.

3.1 Introduction.

Chapter 2 outlined the different life cycles that paedogenetic Cecidomyiidae can follow. Presumably the ability to reproduce by paedogenesis is evolutionarily significant as it is this which, in the wild, allows these insects to exploit transient resources to the full, while in the case of cultivated mushrooms, it enables the insects to achieve large populations and to reach pest status very rapidly.

Two factors which are important during development for cecids are: the sex of progeny produced and their developmental pathway, whether it be to continue to reproduce asexually in the larval form or to pupate and produce adults.

The literature describes both of these factors in detail (Wyatt 1961, Camenzind 1962, and Went and Camenzind 1980). Many authors document the importance of environmental factors in controlling both the sex of the progeny and their developmental fate (Harris 1923, 1924 and 1925, Wyatt 1961 and Camenzind 1962).

3.1.2 Sex determination.

The occurrence of males in populations of paedogenetic cecids is described as being very unusual (Harris 1924, Camenzind 1962). No males have been seen in the cultures at Bath (Chapter 2).

Almost without exception, sex determination in insects is under strict genetic control. As in most other animals it is determined by the presence or absence of sex chromosomes. In most insects, including Diptera, females are homogametic and have

two X, or sex, chromosomes, while the males are heterogametic having either only one sex chromosome (XO) or having one sex and one other different chromosome (XY). However in some insects (e.g. the Lepidoptera), the reverse is the case, and it is the female which is the heterogametic sex (WZ), while males have a pair of identical (WW) sex chromosomes.

In some insects including the Hymenoptera sex is determined by a haplo/diploid mechanism with the females being diploid and the males haploid (Borror, DeLong and Triplehorn 1981). This is commonly (but not exclusively) associated with sociality (Trivers and Hare 1976).

However, unlike the case in mammals, in almost all the cases in which the matter has been investigated, insects lack sex hormones, and there are no 'secondary' sexual characters, gender differences being programmed directly by the chromosomal makeup of individual cells. This is shown by the existence of sexual mosaics or gynandromorphs, in many species of insects.

The single case where it has been claimed that the sex of an insect is determined secondarily, by an endocrine mechanism, is that of the glow worm, *Lampyris noctiluca* (Naisse 1965 and Nijhout 1994) where it has been shown that sex determination is due to an androgenic hormone secreted by the testis.

In parthogenetic insects, such as the Cecidomyiidae, sex determination is usually by the hetero/homogametic mechanism. If males are produced it is due to the loss of an X chromosome to give (XO). The fusion of two nuclei to give XX will give female insects (Went and Camenzind 1980, Borror, Triplehorn and Johnson 1989).

3.1.3 Factors influencing sex determination.

Presuming that a hetero/homogametic mechanism is used to determine male or female insects in *Mycophila speyeri* and *Heteropeza pygmaea* and that there are two sexes, what causes the selection of males or females during development? In his review Matuszewski (1982) outlines the environmental conditions which various authors have singled out as being responsible for sex determination.

Went (1975) describes how, when grown in laboratory culture, males or male-producing females were only seen when populations were high. Wyatt (1961), Camenzind (1962) and Went (1975) all attribute sex determination to nutritional variation.

Camenzind (1962) manipulated the malt content of the agar on which fungus was grown in order to control the sex ratio in *H. pygmaea* fed on *Peniophora albula* (= *Corticium evolvens).* When the fungus was grown on 2% malt-extract agar male insects were produced, but when the fungus was grown on 0.5% malt-extract agar the population consisted only of females.

Wyatt (1961) describes how he could alter the sex of a larval population by growing the insects in question on plates containing different species of fungus. When an unidentified species of cecid was cultured on plates containing *Agaricus bisporus*, the insects reproduced paedogenetically until the plates became contaminated with a fungal contaminant *Chaetomium* sp. Wyatt claimed that this contamination led to the production of three male individuals which went on to produce adult males, although apparently these were infertile.

Went (1975) claims that in *H. pygmaea* overcrowding leads to a lack of food, which in turn gives male larvae. It is questionable, however, whether overcrowding is the sole cause for the switch in this case, Went goes on to say that placing the insects on a plate

of older fungal mycelium has the effect of producing males. Using the culturing techniques that Went describes, it is impossible to consider overcrowding and fungal age in isolation; when the plates have become overcrowded the fungus has aged and other conditions, such as the amount of fungal and insect waste products in the culture, will also have altered.

Confusion exists as to how the sex of populations of paedogenetic Cecidomyiidae can be differentiated and manipulated. Went's (1975) paper on *H. pygmaea* is not simply about the production of males, but also about the production of pupae. The sex of larval insects can only be distinguished after pupation. Harris (1924) states that in the larval form of the cecid *Miastor metraloas* the two sexes are indistinguishable unless they pupate and produce male and female adults.

Harris (1924) raises doubts as to whether males can be produced during the paedogenetic cycle. He states that in *Miastor metraloas* males can only be produced from imago eggs. If so how are these eggs produced unless it is by parthenogenesis?

If this is the case then the developmental pathway taken by these insects is doubly important as it not only determines the method of reproduction but can also have an effect on the sex of the population.

Whichever environmental factor is responsible for the switch there must also be control at the physiological level. The environmental factor must trigger a response within the insect to cause the developmental change to occur. Went and Camenzind (1980) describe how the sex of *H. pygmaea* larvae emerging from eggs cultured *in vitro* is dependent on the sex of the insect used to donate the haemolymph in which they are cultured; this suggests that a hormonal trigger is responsible.

This does not necessarily imply, however, that sex determination in *H. pygmaea* is not chromosomally determined, since a maternal sex determining hormone might act by causing selective chromosome elimination during development. Such chromosome elimination is a feature of early development in cecids (Chapter 1, Schüpbach and Camenzind 1983).

3.1.4 Developmental fate.

It is clear from the literature that not only the sex but also the developmental fate of laboratory-reared cecids is determined by the conditions experienced by the paedogenetic "mother" insect in *H. pygmaea* (Went and Camenzind 1980). In the insects' natural environment seasonal variation plays an important role.

Harris (1925) working on *Oligarces* sp. describes seeing many pupae in cultures found in the field in the summer, in comparison to those studied in the spring, autumn and winter. He surmises that this is due to the fact that a switch in developmental pathway away from that of paedogenetic development to the adult cycle is directly due to overcrowding and that this is experienced in the summer due to increased reproductive rate. His study concentrated on the effects of light, temperature and overcrowding. The last of these factors was the only one which he found would cause a switch in developmental pathway. He found that light had no effect on developmental fate, and that temperature had no significant effect other than to speed up the rate of reproduction.

In earlier experiments Harris (1923) had noticed that if an isolated piece of wood, containing both fungus and cecids, was sealed in a metal box there was an increase in the number of pupating insects. Although Harris attributed the switch to overcrowding, it could also have been due to an exhaustion of food supplies, a build up of toxins, a change in the fungus or any combination of the other factors associated with an aging culture.

3.1.5 Introduction to experiments.

During routine cultures carried out at Bath on *M. speyeri* and *H. pygmaea* normally only paedogenetic larvae were seen. Insects have been seen to alter their pathway to produce pupae and adults when cultures have deteriorated and become old but this has not been a phenomenon which has been easy to control. In all cases where these adults have been examined, they were all female.

In this Chapter I describe experiments which were undertaken to see what factors caused this switch in development, and whether it would be possible to manipulate the environment of cultures in order to produce insects destined to pupate and to produce adult flies, or to produce males. This would be a very valuable research tool which could be used to further understand reproduction in these insects, and what might ultimately be of applied significance.

3.2 Materials and Methods.

3.2.1 The influence of nutrition on sex determination in *M. speyeri.*

This experiment was similar to the one described by Camenzind (1962) to see whether insects of a different sex were produced when insects were grown in conditions of varying nutritional quality. Any effects that the treatment had on developmental pathway were also noted.

Insects were reared on *Chondrostereum purpureum* as described in section 2.2.1. Three sets of agar plates containing different concentrations (0.5, 2.0 and 3.0%) of malt-extract agar were used. The numbers of individual offspring produced by the paedogenetic larva reared on each malt concentration, the time taken for each generation to be produced and the developmental fate of the insects left on deteriorating cultures were all noted. After each generation one of the offspring from each plate was removed and placed on a new, pre-inoculated plate. The experiment was monitored over 5 generations.

3.2.2 Culture of *M. speyeri* on defined medium.

Leatham's medium (Leatham 1983) was selected as a suitable defined substrate for *C. purpureum*. The medium consists of mineral, trace element and vitamin solutions combined with glucose, glutamic acid, glucuronic acid, magnesium salt and potassium salt. These are made up to 1 litre with distilled water and 25g number 2 agar (LABM). The carbon:nitrogen ratio was 35:1 and the final pH was adjusted to 5.7. Plates were prepared as in section 2.2.1 and were inoculated with *C. purpureum*. One paedogenetic larva from stock culture was then introduced to each plate. One insect was also placed onto each of a set of 2% malt-extract agar plates in order to compare any differences in development. The numbers of insects produced per generation, the sex of these individuals and the fate of larvae left on deteriorating plates were all noted. After each generation the insects were moved to a new set of fungal plates and the experiment was continued for 3 generations.

When differences were seen between the insects grown on malt-extract plates and those grown on the defined medium a further set of experiments was planned to monitor whether it was the lack of malt-extract in the plates that caused the differences or whether it was the ingredients in the Leatham's medium.

Six sets of agar plates containing the following types of media were made: Leatham's medium, as described above, Leatham's medium additionally containing; 0.25, 0.5, 1.0 and 2.0 % malt-extract and normal 2% malt-extract agar. Five plates of each were inoculated with *C. purpureum* for the first generation and after three days of mycelial growth all had one *M. speyeri* larva placed on them. The number of offspring produced per generation, the time taken for each generation and the fate of insects left on deteriorating plates were all noted. The experiment was followed for three generations.

3.2.3 The effect of temperature on the growth of *M. speyeri.*

The normal culture temperature for *M. speyeri* is 24°C. An experiment was carried out to confirm the results of Harris (1923) on *Miastor metraloas* that temperature does not affect the developmental fate of cecids. Three sets of inoculated malt-extract agar plates each containing 5 replicates were put at three different temperatures: 15, 24 and 30°C. They were first cultured over a period of one week. At the end of this period any differences between the plates were noted and they were then left for a further week.

3.2.4 The effect of fungal species of the growth and development of *M. speyeri.*

Paedogenetic cecids, including *M. speyeri*, are commonly found in the field beneath the bark on decaying wood, where they encounter many fungal species. Wyatt (1961) stated that the presence of different fungal species could affect the sex of the progeny produced by a paedogenetic mother. An experiment was carried out to grow *M. speyeri* on different fungal species and to monitor any effect it had on reproduction or the sex of offspring.

Nine cm Petri dishes containing 2% malt-extract agar were inoculated with one of the following fungal species: *Hypoxylon fuscum, Chondrostereum purpureum, Vuilleminia comedens* and *Corticium evolvens*, 5 plates of each. The mycelium was allowed to grow on each plate for 5 days. This was to ensure coverage of the plates even by the slowest growing species. After 5 days one paedogenetic *M. speyeri* larva was placed onto each plate. The numbers produced per generation, the time taken for each generation to be produced and the sex of adult flies produced on deteriorating plates were all noted. When each new generation was produced, one of the offspring from each plate was transferred to a new set of pre-inoculated plates. The experiment was continued over 3 generations.

The four species chosen were all from the phylum Basidiomycota with the exception of *H. fuscum* which is from the phylum Ascomycota. They are all commonly found in the field and are commonly grazed by the Cecidomyiidae. *C. evolvens* (formerly *P. albula*) is the fungus used for laboratory culture of cecids by many authors. *C. purpureum* is the fungus used in this study and was incorporated into this experiment as a control against which to monitor the effects of the other species.

3.2.5 The effect of crowding on the growth and development of *M .speyeri.*

Three experiments were designed to investigate the effect of crowding on populations of *M. speyeri.* In the first experiment fungal cultures of *C. purpureum* were established on agar plates, as described in Section 2.2.1. On one set of 5 plates one insect was used to begin a colony, on another set 10 larvae and on a third set of 5 plates 30 *M. speyeri* larvae were used. It was hoped that on the plates containing more insects, crowding would occur more rapidly and a switch in development would be seen after a shorter period of time. The time taken for signs of pupation to occur was noted.

The second experiment was designed to see whether it was the age of the fungus in a crowded culture which triggered the switch, away from a paedogenetic cycle to the production of adults, rather than the density of the insects *per se*. Three sets of 5 plates containing 2% malt-extract agar inoculated with a culture of *C. purpureum* were made as described in Section 2.2.1. One insect was placed on each of the first set of plates when the fungus was 3 days old. When the fungal culture was 7 days old one insect was placed onto each of the second set of plates. At 14 days one insect was placed on each of the final set of 5 plates. Three weeks after insects had been introduced to each plate, the plates were assessed for any evidence of pupa or adult production.

During the second experiment it was observed that placing larvae onto ungrazed, two week old plates did not replicate the conditions found on a 14 day old culture which has been grazed by a larval population. The mycelium which was being produced on the 14 day old fungal cultures was tougher and more hydrophobic with tougher cell walls. The insects on these plates were dying because they were unable to feed on the mycelium. The following experiment was designed to replicate the fungal conditions found on a two week old plate without using insects. Two % malt-extract agar plates were made as described in 2.2.1. Before they were inoculated with the fungus *C. purpureum* the agar were overlaid with sterile cellophane discs. This allowed the fungus to grow normally on the plates. After 14 days the cellophane and the fungal mycelium was peeled off before the plates were re-inoculated with *C. purpureum*.. When the new fungal culture was three days old one insect was placed onto each plate. After 21 days the production of pupae and adults on these plates were compared to fresh plates on which the fungus had been allowed to grow for 3 days.

3.3 Results.

3.3.1 Influence of nutrition on sex determination in *M. speyeri*.

To determine whether larval nutrition was a factor influencing the reproductive potential, developmental pathway and sex determination of *M. speyeri*, the host fungus *C. purpureum* was grown on agar plates containing 0.5, 2.0 and 3.0% malt-extract. The results are shown in Table 3.1.

Table 3.1 Results from experiment to examine sex determination by nutritional variation.

% Malt Concentration	Mean number per generation ± SE	Mean gen. time (days) ± SE	Number of plates on which pupation occurred	Sex of imagoes produced	n
0.5	21.9 ± 4.8	5.3 ± 0.88	2	female	15
2.0	19.5 ± 3.2	5.05 ± 0.8	1	female	15
3.0	23.1 ± 3.8	5.85 ± 0.89	3	female	15
	f = 1.92	f = 1.85			
	$p = 0.15^{NS}$	$p = 0.16^{NS}$			

All of the first-generation insects produced on all three treatments were themselves able to reproduce paedogenetically. Since there were no significant differences between any of the parameters measured in successive generations of insects in the same treatment categories, the results for successive generations of the same category were pooled giving n=15 for each treatment. There were no significant differences in the number of insects produced per generation ($p = 0.15$) or in the time taken for the insects on different media to produce the next generation ($p = 0.16$, Table 3.1). Only a

small number of plates went on to produce adult insects and there were no significant differences in the number of plates on which pupae and adults were eventually seen. As in 2.3.2 all of the adults were female.

One noticeable difference between the cultures was the difference in the growth habit of the fungal mycelium on the different media. The fungus on the 0.5% malt-extract agar covered the plate far more rapidly and grew far more sparsely than on the 2.0 and 3.0% treatments. This was presumably because of the reduction in nutrients available to the fungus. Although this resulted in a reduction in the fungal biomass available as food for the larvae on the 0.5% treatment, there was no effect on the cecid population during the period of the experiment.

3.3.2 Culture of *M. speyeri* on defined medium.

To test whether a less complex growth medium for the fungus would have an effect on the generation time and the numbers of insects produced per generation. *M. speyeri* larvae were cultured on *C. purpureum* grown on Leatham's defined medium. The results are shown in Table 3.2.

Table 3.2 Culture of *M. speyeri* on defined medium.

Treatment	Mean number per generation ± SE	Mean generation time ± SE	n
Leatham's medium	14.13 ± 5.8	5.73 ± 0.96	15
2% malt-extract agar	20.08 ± 1.19	5.3 ± 0.65	12
	f = 3.7	f = 1.51	
	$p = 0.06^{NS}$	$p = 0.22^{NS}$	

As in 3.3.1 the data for the 3 successive generations were pooled for each treatment. *M. speyeri* larvae did not grow and reproduce as well on Leatham's medium as on the conventional substrate (Table 3.2). The number of *M. speyeri* larvae per generation was reduced but was not found to be significant ($p = 0.06$). Although the larval generation time was slightly longer, this effect was not statistically significant ($p = 0.22$). In addition to the differences between the treatments shown in the table, there was a marked difference in the appearance of the fungus and the larvae.

The growth of the fungus did not appear to be any quicker on the Leatham's medium but the aerial mycelium did appear to be denser. It was also observed that the *M. speyeri* larvae did not thrive on the Leatham's plates; instead the progeny produced were very much reduced in size and showed a slightly longer generation time. There was a difference in developmental fate of these insects when the plates deteriorated with increasing age. Whereas on the malt plates the populations eventually went on to produce larvae which in turn became pupae and then adults, on the Leatham's media the insect populations died out without producing pupae.

This could be attributed to the type of fungal growth seen on the plates. There was an increase in the amount of aerial mycelium produced, perhaps due to the increase in the carbon:nitogen ratio. This aerial mycelium, which is composed of non-assimilative hydrophobic hyphae, may be tougher and is not as nutritious to the cecid larvae as is the softer, assimilative mycelium produced on the malt-extract agar plates. This difference may be attributed to the fact that Leatham's medium was originally developed to induce fruiting in the basidiomycete *Lentinus edodes* (Leatham 1983).

To test whether the addition of malt-extract to the Leatham's medium could alter the results seen in Table 3.2 varying concentrations of malt (0.25, 0.5, 1.0, 2.0%) were added to the agar. The results are shown in Table 3.3.

Table 3.3 Culture of *M. speyeri* on Leatham's media with malt supplement.

Media	Mean no. of insects per generation ± SE	Mean generation time ± SE	n
Leatham's	8.8 ± 2.6 [a]	5.7 ± 0.3	30
Leatham's + 0.25% ME	14.15 ± 1.9 [b]	4.6 ± 0.4	30
Leatham's + 0.5% ME	14.15 ± 1.9 [b]	5.7 ± 0.54	30
Leatham's + 1.0% ME	12.13 ± 1.8 [b]	5.9 ± 0.54	30
Leatham's + 2.0% ME	14.2 ± 2.31 [b]	5.0 ± 0.31	30
2% malt-extract	20.5 ± 2.6 [b]	5.2 ± 0.14	30
	f = 3.42	f = 1.36	
	p = 0.0272***	p = 0.272[NS]	

The results in Table 3.3 support the findings from above; that there is a smaller number of insects produced per generation on Leatham's medium plates in comparison with malt-extract agar plates. There was a significant difference in the numbers of insects produced on the different treatments (p = 0.027). When the means were compared it was clear that it was the number of offspring produced on the Leatham's medium which was significantly different to those produced on the media containing malt-extract (signified by [a] on Table 3.3). Larger numbers of insects were produced per generation when the Leatham's medium was supplemented with malt-extract but these did not match the numbers produced on malt-extract agar alone.

The appearance of the fungal mycelium on the plates was similar to that seen in the previous experiment. On the plates containing Leatham's medium there was more aerial mycelium in comparison to the assimilative mycelium seen on the malt-extract plates.

On the plates containing malt-extract agar the deteriorating cultures contained pupae and female adult insects. The plates containing Leatham's media did not show this switch; this was due to the death of the insect larvae on these plates.

It is unclear whether the results above show that an increase in the C:N ratio in Leatham's medium, causes changes in the fungal mycelium which makes it less nutritious to the insects in some way or whether there is some component of the defined medium which is deleterious to the cecids. These hypotheses are in any case not mutually exclusive. The marked increase in cecid numbers with only 0.25% malt-extract suggests that Leatham's medium lacks something which is provided by malt-extract. On the other hand, even with 2% malt-extract, the presence of Leatham's medium was associated with a significant decrease in cecid numbers compared with the 2% malt-extract alone.

3.3.3 The effect of temperature on the growth of *M. speyeri.*

To test whether temperature had an effect on the developmental fate, the generation time or the numbers of larvae produced per generation 3 sets of insects were cultured at different temperatures (15, 24 and 30 °C), the results are shown in Table 3.4.

Table 3.4 Culture of *M. speyeri* at different temperatures.

Temperature (°C)	**Mean number of insects produced ± SE**	**n**	**Mean generation time (days) ± SE**
15	16.0 ± 2.64	3	7.33 ± 0.3 [a]
24	15.66 ± 3.17	3	4.66 ± 0.32 [b]
30	15.66 ± 1.20	3	4.0 ± 0.0 [c]
	f = 0.0059		f = 42.0
	p = 0.99^{NS}		p = 0.0002***

No difference was seen in the developmental fates of insects produced at different temperatures. As observed by Harris (1924) with *Miastor metraloas* the only effect that increasing the temperature had was to significantly decrease generation time ($p = 0.0002$). When the means were compared it was found that the generation time at each temperature was significantly different to the others.

At very high temperatures the insects died of starvation after the mycelium failed to grow any further. If plates were put at extremes of low temperature the fungus ceased to grow. At temperatures below 8 - 10 ^{o}C the development of both fungus and insect were very slow.

3.3.4 The effect of fungal species on the growth and development of *M. speyeri.*

To test whether the food source available to the insects had any effect on reproductive development larvae of M. speyeri were cultured on four different species of fungus: *Hypoxylon fuscum, Chondrostereum purpureum, Vuilleminia comedens* and *Corticium evolvens*. The results are shown in Table 3.5.

Table 3.5 Culture of cecid populations on different fungal species.

Fungal species	Mean no. insects per generation ± SE	No. of lines which died out	Mean generation time ± SE	n
V. comedens	7.21 ± 0.9 [a]	2	10.6 ± 1.26 [a]	14
H. fuscum	6.8 ± 0.88 [a]	1	9.5 ± 0.69 [a]	15
C. purpureum	12.4 ± 0.79 [b]	0	5.72 ± 0.29 [b]	28
C. evolvens	11.16 ± 1.37 [b]	2	4.94 ± 0.14 [b]	16
	f = 8.15		f = 26.5	
	p = 0.0001***		p = 0.000***	

Data for 3 or more successive generations were pooled to produce the mean values shown in the table. Only those cases where the single larva introduced onto the plate survived to produce viable offspring were included in this analysis. The table also shows the number of lines that died out.

The results show that there was a significant difference between the numbers of insects produced ($p = 0.0001$) and the time taken for each generation to be produced ($p = 0.0000$) depending upon the species of fungus on which the larvae had been grown. When the means of the data were compared it was found that those *M. speyeri* larvae grown on *C. purpureum* and *Corticium evolvens* produced larger numbers of offspring per generation and had a shorter generation time to those grown on *Hypoxylon fuscum* and *Vuilleminia comedens.*

Table 3.5 does not reflect all the effects seen on the experimental plates. The fungal mycelium on the *Vuilleminia comedens* and the *Hypoxylon fuscum* plates was very slow growing and the mycelium was very fine. The insects did not thrive on these

cultures and the individuals which were produced were paler in colour, smaller and less active than those on *Corticium evolvens* and *C. purpureum*. The size of the larvae on *Vuilleminia comedens* and the *Hypoxylon fuscum* did increase slightly over the three generations and the quality did improve but they were still smaller paler and less active than the larvae produced on the plates of the other two fungal species, even after the third generation. The mycelium of *Hypoxylon fuscum* produced regions of darkening within the agar substrate; this is a characteristic response of this fungus to stress.

The *M. speyeri* larvae produced on *Corticium evolvens* were particularly large and healthy. The fat bodies of the paedogenetic individuals were deeper orange in colour when compared to those raised on *C. purpureum*. This may be due to the fact that the hyphae of *Corticium evolvens* characteristically contain a large amount of lipid. This can actually be seen in the mycelium in droplets. It is possible that the insects feeding on this species are able to accumulate a larger amount of stored lipid in their fat bodies compared to those feeding on other fungal species.

None of the fungal species used caused a switch in the developmental pathway from paedogenetic to adult. In some plates from all species a certain amount of pupation and production of adult flies was seen. More pupation was seen on plates containing *C. purpureum* and *Corticium evolvens* but this was probably due simply to the fact that these plates contained more larvae. All of the adults produced were female; many laid eggs but these did not hatch and no larvae were produced.

Populations of cecid larvae are found on all four fungal species in the field (C. Taylor, University of Bath - personal communication). This does not explain why *M. speyeri* thrived on only two of the species in the laboratory. It may simply be that under laboratory conditions, in the absence of other limiting factors, the growth habits of *C. purpureum* and *Corticium evolvens* are well suited to the feeding methods of *M. speyeri*, and that the insects are consequently able to derive more nutrition from these

species and to sustain a higher rate of reproduction. Alternatively it may be that in laboratory culture rather than in the field *Hypoxylon fuscum* and *Vuilleminia comedens* produce metabolites which inhibit the growth of *M. speyeri* in some way.

3.3.5 The effect of crowding on the growth and development of *M. speyeri.*

To test whether crowding has an effect on the method of reproduction used by *M. speyeri*, the number of larvae used to begin a culture was varied and the time taken for pupation to occur was noted. The results are shown in Table 3.6.

Table 3.6 The occurrence of pupation on crowded plates.

N° of larvae used to start culture	**Time taken for pupation to occur in 3 or more plates (days)**	**n**
1	16	5
10	18	5
30	15	5

The results shown in Table 3.6 do not show any difference between the time taken for pupation to occur on plates that had been artificially crowded compared with plates that were initially colonised by one paedogenetic larva of *M. speyeri.* This may have been due to the fact that the numbers of larvae used to induce crowding were not sufficiently high.

After one generation the plates that had been colonised with a single larvae would have contained around the same number of larvae as those which had begun the experiment with 10 individuals. It may be that crowding *per se* does not cause the switch between a paedogenetic cycle and the production of adults and that there are different factors

which occur in a culture over time, such as the age of the fungal culture, which determines whether a switch takes place.

To test whether the age of the fungal mycelium did play a role in the developmental fate of paedogenetic *M. speyeri* larvae, larvae were placed on fungal cultures of differing ages and the numbers of plates showing signs of adult or pupa production were noted after 21 days. The results are shown in Table 3.7.

Table 3.7 The effect of fungal age on the development of *M. speyeri.*

Age of fungal culture (days)	**N° plates where pupation was seen after 21 days**	**N° plates on which insects died out**	**n**
3	3	0	5
7	4	0	5
10	0	5	5

Table 3.7 shows that the age of the fungal culture did not have an effect on the number of plates on which pupation was seen. It did, however, have an effect on the larvae. Those which were placed on the plates containing fungus which was 10 days old did not thrive. They did not appear to feed successfully on the mycelium which was tough and hydrophobic. They failed to grow and subsequently died.

It was recognized that a 10 day old 'ungrazed' culture of *C. purpureum* did not replicate the conditions of the fungus found on a plate with a 10 day old 'crowded' fungus and insect colony on which pupation may be starting to occur.

To test whether on a 'crowded' plate it may be the presence of an 'old' grazed fungal culture which causes the switch between paedogenetic development and the production of pupae and adults the following experiment was designed. Insects were placed either onto plates with a fresh fungal culture or onto plates with new fungal cultures inoculated onto agar which had been inoculated, allowed to grow and had then had the fungal mycelium removed. The results are shown in Table 3.8.

Table 3.8 The effect of 'old' fungal cultures on the development of *M. speyeri.*

Fungal treatment	**N° plates showing pupation after 21 days**	**n**
Fresh culture	3	3
Cellophane/ regrown culture	3	3

Table 3.8 shows how there was no difference in the number of plates showing pupation when fresh fungal cultures are compared with cultures on which fungus has been previously grown, removed and re-inoculated. The fungal mycelium which was produced on the plates which were re-inoculated was very similar in appearance to the plates on which the fungal culture was fresh.

The results of these three experiments suggest that perhaps larval density is not the sole reason for a switch from a paedogenetic cycle to the production of adults and that other factors may be involved. They do, however, suggest that it may not be the age of the fungal culture which is responsible. It is clear that the conditions which are present on an 'old' plate with ageing fungal and insect cultures are very difficult to recreate in the laboratory in isolation.

3.4 Discussion.

The experiments described in this chapter were designed to investigate the possible roles of environmental variables in controlling this developmental decision. The results confirm the results of Harris (1923), that temperature has no effect on the developmental fate of *M. speyeri* other than to accelerate larval development. The results however, fail to support the findings of Wyatt (1961) and Camenzind (1962) who claimed that nutritional variation can affect the decision to pupate and sex determination. Crowding has frequently been discussed by earlier authors as a factor in the control of pupation and sex determination.

First, temperature. Harris (1923) actually observed seasonal variation in a cecid colony composition. He suggests that in the field temperature plays an important role in regulating the reproductive rate of paedogenetic cecids and therefore the extent to which overcrowding and a subsequent developmental switch occurs. He says that the rate of reproduction is most rapid in the summer due to the higher temperatures and that therefore the occurrence of adult flies during this season is common. Here no correlation between temperature and pupation or sex determination was found. The present experiments suggest that it is far more likely that it is the poor growth of fungal mycelium in the summer which leads to a decrease in food quality and triggers a switch in development in order to facilitate dispersion.

During this study, the production of pupae and adults has always occurred during routine culture on 'old' plates (which may also be described as 'degenerating cultures of fungus'). This has been described by many other authors including Wyatt (1961) and Camenzind (1962). Many of these authors have assumed that this is a response to crowding, but it is evident from this study that a number of other factors in a crowding situation also differ from a 'normal culture', including the age and quality of the fungal mycelium. Three experiments were performed to test these variables (Section 3.2.5).

In brief, although some of the treatments led to poor growth and development of the insects compared to 'normal' culture conditions, in no case was it found that the treatment reproducibly induced pupation.

Crowding has frequently been asserted to play a role in triggering the switch away from the paedogenetic cycle to the production of adults (Harris 1923, Wyatt 1961 and Camenzind 1962) in culture. It is however doubtful whether crowding of larvae can in fact be studied in isolation from all of the other environmental factors.

In the experiments described here, it is extremely probable that a number of other important variables were changed in addition to larval density: a reduction in food quantity, a build up of insect toxins and metabolites, an aging of the fungal mycelium which includes a build up in fungal metabolites and a general reduction in food quality, and an alteration in the quality of the plates including nutritional water content, would all have been expected to occur at the same time.

Overcrowding is not the single most important factor *per se*. Time is very important as a mature culture with the qualities described above is only obtained after a certain amount of time has elapsed.

Mycetophagous insects are very responsive to changes in fungal quality (Boddy, Coates and Rayner 1983). Went (1975) describes seeing males when he cultured *H. pygmaea* on old fungal cultures of *P. albula* (= *C. evolvens*). He attributes this to an alteration in fungal quality. A similar phenomenon has been seen during the course of this study. When insects were put onto plates of Leatham's medium onto different fungal species and onto older fungal plates a qualitative change was seen in the insects. This did not involve a production of males but simply saw the production of underdeveloped individuals. A fungus alters its metabolism and hyphal composition as

it ages, increasingly producing aerial mycelium which have tougher hydrophobic cell walls and which probably provide a poorer food source for the insects.

An important conclusion of the experiments described in this chapter is that the insects kept in culture at Bath do not produce males, or if they do they produce them extremely rarely. The occurrence of males cannot easily be manipulated by environmental factors. It is very difficult to highlight one particular environmental factor which is responsible for producing a switch in developmental pathway. It is thought that it is a number of factors acting simultaneously which can trigger the switch. These include a reduction in fungal quality and quantity, a deterioration in the quality of the plate and an increase in the numbers of insects on the plate. All of these factors change with time.

Chapter 4

Endocrine Control of Paedogenesis.

4.1 Introduction.

4.1.1 Insect developmental hormones.

Hormones are responsible for controlling many aspects of development, including reproduction, moulting, behaviour, diapause and metabolism. In all insects the two major types of developmental hormone are the juvenile hormones (JH) and the ecdysteroids. Both have been widely studied for many years and the results have been reviewed by many authors (e.g. Wigglesworth 1970, Highnam and Hill 1978, Riddiford 1985, Nijhout 1994).

Hormones can be classified depending on the type of physiological effect that they have. Regulatory hormones, which are responsible for the control of metabolic and homeostatic functions, have constantly fluctuating levels. By contrast, developmental hormones act as triggers for major irreversible physiological changes, and are often only produced when these changes are made. The same hormone can have different effects on the same tissue or organ depending on the stage of the insect (Nijhout 1994).

4.1.2 Production of hormones.

The JHs and the ecdysteroids are produced by glandular tissues. The ecdysteroids are mainly produced in the prothoracic glands, but they are also produced by the ovaries and testes in some insects (Wigglesworth 1970 and Nijhout 1994). The structure of the prothoracic glands varies between different insect orders, but in general the prothoracic glands takes the form of connected strands of secretory cells situated within the prothorax. In the higher Diptera the prothoracic gland is fused with the corpora cardiaca and the corpora allata to form what is known as a ring gland which is close to the midgut (Highnam and Hill 1978, Nijhout 1994).

The prothoracic glands do not store ecdysteroids; instead the hormones are released as they are produced and the ecdysteroid titre in the insect is therefore largely dependant on the rate of production, although the rate of hormone destruction may also play a part in determining the level of active hormone. In most insect orders the prothoracic glands undergo cell death at metamorphosis, and where the adult insects relies upon ecdysteroids for control of reproduction, these are produced by the ovaries and testes (Nijhout 1994).

The JHs are produced by the corpora allata (Wigglesworth 1970). They usually comprise a pair of small glands found in the neck region. Like the ecdysteroids the titre of JHs in the haemolymph is directly dependant upon the rate of production as no hormone is stored. Although the corpora allata become inactive in terms of JH production when the insect undergoes metamorphosis the glands resume activity in the

adult stages in order to provide the JH needed for reproduction (Highnam and Hill 1978, Nijhout 1994).

Insects also possess a neuroendocrine system; neurosecretory cells can be found mainly in the brain, but can also be seen in all the ganglia of the central nervous system. They produce small polypeptides or neurohormones. These neurohormones are not directly released by the neurosecretory cells but are transferred via the cell axons to neurohaemal organs which release the product into the haemolymph. The major neurohaemal organ for neurohormones produced in the brain is the corpora cardiaca (Nijhout 1994), but the corpora allata also act as neurohaemal organs, releasing hormones produced in the brain into the haemolymph (Highnam and Hill 1978, Nijhout 1994).

The production of developmental hormones by the prothoracic glands and the corpora allata is controlled in turn by the presence of other hormones within the insect's system (Wigglesworth 1970). It is not appropriate to review this topic in depth, but briefly, the production of ecdysteroids by the prothoracic glands is regulated by a neuropeptide hormone, prothoracicotrophic hormone (PTTH) released from the corpora cardiaca or the corpora allata. The chemical nature of this hormone has long resisted elucidation. It has recently become clear that PTTH is a member of a protein hormone superfamily that includes growth hormone (Noguti *et al.* 1995).

The production of JHs by the corpora allata, on the other hand, seems to be regulated by a number of neurohormones, the allatotropins and the allatoinhibins. These are

smaller peptides which are probably supplied to the corpora allata directly through its innervation from the brain. They are numerous and it is fair to say that their functions are not well understood (Woodhead *et al.* 1989, Duve *et al.* 1993).

The production of the trophic hormones that regulate the endocrine system is dependent on information that the brain and other neurosecretory regions receive from the external environment and internal chemical triggers (Bollenbacher and Granger 1985). It is also true to say that insect tissue will only produce a response to certain hormones if the correct receptors are present. At certain times during the life cycle tissues are not responsive to the presence of some hormones. This was demonstrated by Reynolds, Taghert and Truman (1979) in their work on the responsiveness of *Manduca sexta* to bursicon and eclosion hormone.

Hormone actions in cells may be placed in two categories. Peptide hormones cannot pass through the cell membrane; they bind to receptors on the cell's surface and this causes a change to occur in the receptor. These changes cause the production of secondary messengers which elicit a response in the cell caused by pre-existing enzymes and proteins (Nijhout 1994).

By contrast, when steroid or lipid hormones reach their target tissue or organ they are able to pass directly through the cell membranes where they bind to receptors in the cytoplasm or the nucleus. The receptors in the cytoplasm undergo a change which means that they react differently to other cell constituents. In the nucleus the steroid hormone receptor complex binds to base sequences in the DNA and can initiate the

transcription of certain genes (Jones 1995). The appearance of gene and cell products constitutes what we recognise as the cell's response to the hormone.

4.1.3 Juvenile hormone.

The JHs have many and varied effects on insect tissue and play a part in almost all aspects of development and reproduction (Grenier and Grenier 1993, Jones 1995). Their primary role is thought to be to maintain juvenile (larval or nymphal) characteristics after each larval moult. They have also been implicated in the control of behaviour, diapause, vitellogenesis and many other processes (Wyatt and Davey 1996).

There are 5 different naturally occurring forms of JH. They are terpenoids derived from farnesenic acid. They are all methyl esters and have a terminal or subterminal epoxide group. Some insects secrete only one form of JH and insects from different orders will characteristically produce different forms. JH III is the most wide spread form of JH and has been found in all insect orders studied so far (Grenier and Grenier 1993). The JH molecule is nonpolar and lipid-like; it can act both like a steroid hormone and enter the target cell, and also like a peptide hormone binding to receptors in the cell membrane (Nijhout 1994, Wyatt and Davey 1996). JHs are extremely hydrophobic, stick to many surfaces and are difficult experimental hormones.

Once secreted, JH becomes attached to juvenile hormone binding proteins in the haemolymph. These proteins make the JHs more soluble and protect them from degradation by enzymes present in the haemolymph (Nijhout 1994).

As well as its role as a developmental hormone JH plays a role as a regulatory hormone and can regulate the production of ecdysteroids. It stimulates the production of vitellogenin and, prior to the metamorphic moult, it inhibits the secretion of prothoracicotrophic hormone (PTTH) so that no ecdysteroid is produced until all the JH has been cleared from the insect. This is in order to ensure that the moult will result in the production of an adult and not a further larval instar (Nijhout 1994). It is usually assumed that JH acts in a concentration-dependent manner but that it is simply present in discrete quantities at critical times, but this assumption has not been the subject of specific enquiry.

4.1.4 Ecdysteroids.

The ecdysteroids are known as the 'moulting hormones'. They are developmental hormones which, when titres within the insect rise, trigger the insect to moult. In larvae or nymphs this occurs in the presence of JH a subsequent larval stage is produced. At the final larval stage no JH is present and the presence of ecdysteroids causes the insect to undergo metamorphosis and produce an adult.

Wigglesworth (1970) documents the history of the discovery of the ecdysteroids. Ecdysteroids are sterol derivatives, the most commonly found ecdysteroid is 20-hydroxyecdysone, followed by its precursor ecdysone (Rees 1989).

All insects contain at least two forms of ecdysteroid: usually ecdysone and 20-hydroxyecdysone, initially referred to as α and β. Ecdysone is an inactive hormone which does not cause any of the physiological changes which are normally attributed to moulting. In some insects, ecdysone is the product of the prothoracic glands. In other

insects (e.g. the Lepidoptera, *Manduca sexta*) it is produced by the action of enzymes in the haemolymph on 3-dehydroecdysone which is the steroid actually produced by the prothoracic glands (Nijhout 1994). 20-hydroxyecdysone is produced from ecdysone within the fat body, the gut and the epidermal cells. 20-hydroxyecdysone is the primary active form of the hormone and causes the physiological changes observed during moulting: apolysis, cell division, digestion of old cuticle and production of new cuticle (Riddiford 1985). Some insects use ecdysteroids other than ecdysone and 20-hydroxyecdysone; these will be discussed later.

4.1.5 Hormonal control of reproduction.

Ecdysteroids and JHs are also the principal hormones involved in the control of reproduction (Highnam and Hill 1978). In most insects this occurs in the adult insect after both moulting and metamorphosis have ceased. At this stage the ecdysteroids and the JHs take on very different roles than those that they had during the immature stages.

In the reproduction of insects there are many stages which are targets for control by hormones: the production of vitellogenin by the fat body, the production of the egg follicle, the growth of the oocyte and the uptake of vitellin by the oocyte (Nijhout 1994).

Although the prothoracic glands are no longer present in adult insects, ecdysteroids are produced by the ovaries and by the testes in the case of males. The primary role of ecdysteroids in adults from groups such as the Diptera is the stimulation of the production of vitellogenin or yolk protein; in some other insect groups JH is

responsible (Nijhout 1994). Eggs and embryos can also contain large quantities of ecdysteroids; embryos can moult and produce new cuticles while still in the egg.

Wigglesworth (1970) described the role of the corpora allata and JH in the maturation of eggs in adult *Rhodnius*. JHs play a very important role in egg maturation in many insect groups. They control the production of protein by the fat body for incorporation within the eggs (Grenier and Grenier 1993) and also control sperm production (Highnam and Hill 1978). In some adult female insects the production of glue by the accessory glands to stick the eggs on oviposition to a substrate is controlled by JH.

4.1.6 Insect growth regulators.

Many compounds have been synthesised in the search for novel pesticides and a number have potential value for pest management. One of the groups of pesticides which has received much attention in recent times is the insect growth regulators (IGRs) (Nijhout 1994). These are chemicals that interfere with the insect's development, and can cause mortality. Some of these have a hormonal action.

It was hoped that one of the advantages of using one of the insect's own hormones as a pesticide would be that the target insects would not be develop resistance to these agents, without compromising their own physiology. It was also thought that hormonal insecticides would be specific in their mode of action and have a lower toxicity toward vertebrates than conventional insecticides (Grenier and Grenier 1993).

It was found that naturally occurring JHs could not be used as pesticides because of their instability in the environment. JH analogues (JHa) were developed, which mimic the effects of JH on the target insects. If an insect is exposed to high concentrations of JH or a JHa at critical periods during its development it is unable to metamorphose in the normal way. It can occasionally undergo a supernumerary larval moult but it is usually the case that such moults are not completed and the effect is normally lethal.

Some IGRs such as methoprene share with JH a modified terpenoid structure but lack features such as the epoxide group. Others, although having a similar effect to the JHs, are not chemically similar to the JH molecule; these include phenoxyphenoxy derivatives such as fenoxycarb and pyriproxifen. Some of these compounds can even have greater biological activity than the naturally occurring JHs (Wyatt and Davey 1996). It is supposed that the JHas share with endogenous JHs certain critical features of molecular shape that are essential for biological activity.

The common effect of all the JH agonist type IGRs is that in the laboratory they can restore the functions which are removed when the corpora allata are taken away. In field situations when applied in high doses they can all have additional effects such as the lethal disruption of metamorphosis (Wyatt and Davey 1996). Grenier and Grenier (1993) reviewed the effects of the JH analogue fenoxycarb on pest and non-target insects. They found that it has high JH activity but also other non JH specific effects.

Other IGRs, such as the preocenes, act as JH antagonists. Preocenes act as false substrates for the JH biosynthetic enzymes of the corpora allata. They are converted to

highly toxic cytotoxins that kill the cells of the corpora allata and prevents the secretion of JH, thus provoking premature metamorphosis and so disrupting reproduction. So far no true JH antagonist is known, in the sense of compounds that compete with natural JHs for binding at its receptor, but which do not cause JH-like effects.

Ecdysteroids occur commonly in nature, they can be found in plants such as bracken and yew and act as natural pesticides (Lafont and Wilson 1992). Progress has been slower on developing ecdysteroid pesticides; insects can develop resistance to them quite quickly by utilising the ability that they have for clearing ecdysones between each moult (Wing 1988).

Ecdysteroid analogues do exist, Wing (1988) and Wing, Slawecki and Carlson (1988) studied the effects of the nonsteroidal ecdysone agonist RH 5849 on a *Drosophila* cell line and on larval Lepidoptera. They found that RH 5849 mimicked 20-hydroxyecdysone. When RH 5849 was applied to lepidopteran larvae it caused the premature initiation of moulting which proved to be lethal. In the case of a *Drosophila* cell line, resistant populations of cells were found.

Although insect hormone analogues were thought to be a promising solution to the problems of conventional pesticide use there are problems involved. It has been found that insects can develop resistance to JH analogues just as they can for first and second generation pesticides and that the same problems of non specificity in the field occur and cause problems for beneficials.

It is also difficult to use JH analogues in the field as they rely on a very short time of application. It often also means that the insects are only disrupted after they have finished the larval stage in their life cycle, which is often the time when they are at their most damaging (Grenier and Grenier 1993).

4.1.7 Role of hormones in cecid development.

Little work has been carried out the role of hormones in reproduction in the paedogenetic Cecidomyiidae. Kaiser (1970) studied the hormone producing organs in *H. pygmaea.* He found that a separate corpus allatum and prothoracic gland were present in addition to the central nervous system. This is a similar situation to that found in other Nematocera.

Went (1978) reviews the accounts of other authors who believe that juvenile hormone causes the larvae to reproduce paedogenetically and that ecdysone causes them to produce 'pupa-larvae' which go on to produce adults. Treiblmayer, Pohlhammer, Reiske and Adam (1981) used microlasers to remove the prothoracic glands of *H. pygmaea* larvae which were preparing to pupate. They found that the larvae subjected to this treatment produced eggs, reverted to a paedogenetic form and did not go on to pupate and produce adult flies. They conclude that the hormone which is produced by the prothoracic gland (the ecdysteroid) is not the factor which stimulates egg production.

Using the *in vitro* culture technique described in Chapter 2, Went (1978b) performed experiments to determine the effects of ecdysone and JH on follicle formation in *H. pygmaea*. Went did not achieve the same results as the previous authors. He found that JH completely inhibited follicle development and that ecdysone stimulated development. He concluded that ecdysone titres must therefore be high in the larvae during follicle development and maturation. The follicles in this experiment did not produce eggs which matured fully (c.f. Went 1971) so it is impossible to say whether the stimulatory effects seen by Went would have been associated with paedogenetic or sexual adult reproduction. It is also difficult to say whether the effects were physiological, since the hormones must have been present in very high concentrations (pure chemicals were added to drops of haemolymph). It is also possible that both JH and ecdysone have very important roles at different stages of reproduction and that the application of the hormones in this experiment was mis-timed.

Went, Gentinetta and Lanzrein (1984) did work on *H. pygmaea* larvae in order to determine the ecdysteroid titre during the paedogenetic cycle. During this work they found that paedogenetic reproduction in *H. pygmaea* was retarded if the larvae were placed onto 7 day old mycelial cultures of *Peniophora albula* but that paedogenetic reproduction was 'stimulated' if the insects were placed onto fresh, 3 day old, cultures. They supposed, therefore, that the fungal mycelium may contain ecdysone or 20-hydroxyecdysone when young and tested the mycelium for ecdysone content long with the larvae. Went *et al.* used a combination of thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and radioimmunoassay (RIA) to estimate the levels of ecdysteroids present in the fungus and the larvae.

Went *et al.* (1984) had expected to find high quantities of 20-hydroxyecdysone in young larvae (1 day after emergence) which contained developing follicles. In fact, what he found were very low levels of both 20-hydroxyecdysone and ecdysone in the younger (0 - 3 days post emergence) larvae (0 - 4 ng ecdysone equivalents per g fresh weight) and higher levels of both 20-hydroxyecdysone (18 ng ecdysone equivalents per g fresh weight) and ecdysone (8 ng ecdysone equivalents per g fresh weight) in 5 day old larvae.

It must be emphasised that the titres of ecdysone and 20-hydroxyecdysone found by Went *et al.* (1984) in the larval tissue are very low even for 5 day old larvae. Went *et al.* (1984) do not give clear details of the total fresh weight of insect tissue that was used for each hormone extraction, or the characteristics of the antibody. Without these details it is impossible to gauge the accuracy of the figures given.

In this work Went *et al.* did not take into account the fact that when measuring the hormone titres in the paedogenetic insects they were not simply measuring the titre in the insects of a certain age which were collected but also in the developing embryos and offspring that they contain. High levels of 20-hydroxyecdysone were found in the 5 day old larvae when high levels had been expected in young larvae which contained developing follicles. It is likely that the 5 day old larvae used in these experiments contained maturing embryos which did contain developing follicles and which may well have contained high levels of 20-hydroxyecdysone which would have contributed to the overall titre.

Went *et al.* (1984) did not find any significant quantities of ecdysteroids in the fungal mycelium. The fact that paedogenesis was induced when the insects were placed on younger mycelium was probably due to the positive effect that this food source had on the physiological state of the mother in comparison with the negative effects of being placed on ageing mycelium. The effects of this type of treatment have already been discussed (Section 3.2.5).

4.1.8 Alternative ecdysteroids.

Sterols are very important to insect metabolism; they are required not only for hormone production but also to provide cellular membrane constituents. As insects cannot synthesise the steroid nucleus they rely on dietary sterols for both structure and physiology (Svoboda, Feldlaufer and Weirich 1994).

Both carnivorous and phytophagous insects need to transform their dietary sterols into ecdysone. For most insects the dietary sterol used in the ecdysteroid pathway is cholesterol (Rees 1989). While this sterol is freely available to carnivorous insects it is not a sterol which is readily available to phytophagous insects.

The usual pathway for the production of ecdysone (the most common ecdysteroid) for non-carnivorous insects is the dealkylation of phytosterols (Figure 4.1). Phytophagous insects usually ingest C_{29} and C_{28} sterols such as sitosterol, campesterol and stigmasterol. They then dealkylate the C-24 alkyl group of the sterol side chain in order to produce cholesterol (via various intermediates) (Rees 1985). This conversion

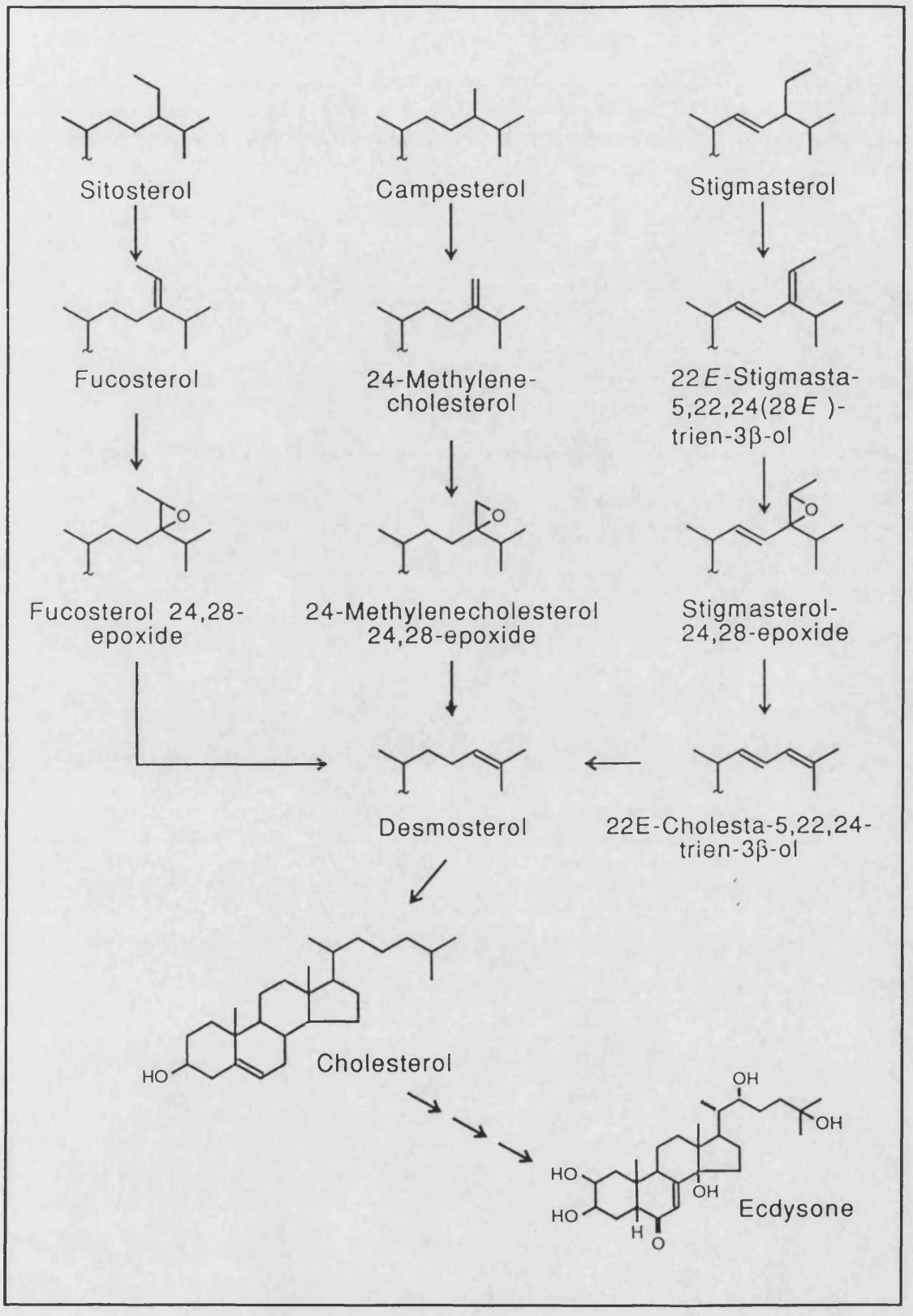


Figure 4.1 A generalized scheme for side chain dealkylation of phytosterols in insects. (from Svoboda *et al.* 1994).

from 24-alkyl sterols to cholesterol provides the precursor to the 27 carbon compounds ecdysone and 20-hydroxyecdysone (Svoboda *et al.* 1994).

Some phytophagous insect species are unable to dealkylate phytosterols and utilise alternative ecdysteroids (Rees 1989). Cholesterol is also absent from fungi just as is the case in plants. Therefore the steroid metabolism of mycetophagous insects is of interest as some are unable to dealkylate higher sterols. The leaf-cutting ant, *Atta cephalotes isthmicola* feeds solely on a fungus that it cultivates and therefore takes in ergosterol as the only dietary sterol (Maurer, Debieu, Malosse, Leroux and Riba 1992). These ants are unable to dealkylate ergosterol and so produce the C_{24} ecdysteroid 24-epi-makisterone A, rather than C_{27} ecdysone (Maurer, Royer, Mauchamp, Porcheron, Debieu and Riba 1991, Maurer, Girault, Larchêveque and Lafont 1993). The leaf-cutting ants provided the first evidence of a functional nervous system completely lacking cholesterol (Ritter, Weiss, Norbom and Nes 1982). As these insects are such specialised feeders it is impossible to change their diet to check for any alternative metabolic pathways (Ritter *et al.* 1982).

The vinegar fly, *Drosophila melanogaster*, which feeds mainly on yeast cannot dealkylate but it can produce C_{27} ecdysone if it is provided with trace dietary cholesterol (Feldlaufer, Weirich, Imberski, and Svoboda, 1995). This is not a characteristic of Diptera since other dipteran species are able to dealkylate; the mosquito *Aedes aegypti* can convert phytosterols to cholesterol (Feldlaufer *et al.* 1995).

The ability to metabolise dietary sterols to those which can be utilised in ecdysteroid production is essential; it enables insects to survive in almost all situations. It is thought that mechanisms of sterol utilisation and metabolism are probably derived from dietary regimens that insects have become specialised for.

Most primitive insects tend to dealkylate dietary sterols to produce cholesterol and subsequently ecdysone. Branching of the phylogenetic tree has resulted in more advanced insects, like *Drosophila,* being unable to dealkylate and being reliant on dietary sterols. Insects which produce alternative ecdysteroids demonstrate a well conserved mechanism for sterol metabolism as they will still do this even if they are given cholesterol in their diet (Svoboda *et al.* 1994).

4.1.9 Aims.

Experiments were carried out to ascertain whether the hormones ecdysone or JH would have an effect on the method of reproduction used by paedogenetic Cecidomyiidae larvae, as the previous work described in Section 4.1.7 gives somewhat conflicting results.

Like other insects, cecids must produce alternative C_{24} or C_{28} ecdysteroids, have a source of cholesterol in their diet, or dealkylate the sterols that are available to them in order to produce cholesterol and then ecdysone. Since, as in the case of the leaf-cutting ant, the only food available to the mycetophagous cecidomyiidae is fungal mycelium a method of extracting sterols from the mycelium was developed in order to

identify them. The sterols present in the insect tissue itself were extracted and identified along with the moulting hormone used by *H. pygmaea*.

4.2 Materials and Methods.

4.2.1 Treatment of insects with hormones and IGRs.

Mycophila speyeri larvae were treated with a number of insect hormone analogues and IGRs. Two % malt agar plates were prepared containing different concentrations of the ecdysteroid analogues RH 5849 (1, 2 - dibenzoyl - 1 - *tert* - butylhydrazine) and RH 5992 (tebufenozide). While the agar was still molten 30, 10, 3, 1 and 0 ppm of the ecdysteroid analogues dissolved in ethanol (a gift from Dr. G. Carlson, Rohm and Haas Co., Spring House, PA, U. S. A.) were added to different sets of plates. The plates were inoculated with the fungus *Chondrostereum purpureum* as described in Section 2.2.1. After the fungus had grown for 3 days a paedogenetic larva from stock culture was then introduced to plates from each set. The number of offspring produced by each paedogenetic larva was noted and the experiment was continued for 3 generations.

In order to monitor the effect of JH on the reproductive ability of paedogenetic *M. speyeri* individual larvae were treated with solutions of pyriproxyfen, a JH analogue (a gift from Sumitomo Co.). Solutions of 30, 10, 3, 1, 0.5 and 0 ppm pyriproxyfen dissolved in acetone were used. Five insects were placed into each of the solutions for 5 minutes before being removed and individually placed onto 2% malt-extract agar plates inoculated three days previously with *C. purpureum*. The reproductive success of the insects was monitored over three generations in terms of the numbers of offspring produced per generation and the time taken for each generation was noted.

A preliminary experiment suggested that the application of JH analogue to the paedogenetic larvae reduced the numbers of offspring produced. The experiment was repeated using JH analogues fenoxycarb, pyriproxifen and JH III (Sigma) in order to establish whether these IGRs would have a similar effect.

4.2.2 Identification of dietary sterol.

In order to identify the sterol present in the diet of the paedogenetic cecid larvae, the following method was used to extract any sterols present in the fungal mycelium.

The fungus *C. purpureum* was grown on agar plates containing 2% malt-extract agar, as described in Section 2.2.1. Sterile cellophane discs were laid over the agar prior to inoculation in order to make harvesting the mycelium simple and in order to prevent the mycelial sample becoming contaminated with the malt agar.

The fungal material was freeze dried, ground using a pestle and mortar and suspended in 20 ml ANALAR ethanol. In order to separate the sterol fraction from the saponifiable fraction the sample was then transferred to a 100 ml round-bottomed flask and 2 ml of KOH in H_2O (stock solution 100g per 100 ml), 0.1 g of pyrogallol (an antioxidant) and anti-bumping granules were added. This solution was refluxed over a boiling water bath for 30 minutes and allowed to cool.

The non-saponifiable fraction was then extracted using redistilled diethyl ether ANALAR grade (BDH); the sample was transferred to a 250 ml separating funnel and 80 ml of redistilled diethylether was added. The funnel was swirled gently and 70 ml

of distilled water was added. The ether fraction was removed and the aqueous ethanol fraction was re-extracted twice more with redistilled diethyl ether.

The diethyl ether fractions were combined and washed with distilled water before being rotary evaporated to dryness at 40^{o}C. The dried fungal sample was redissolved in a small volume of redistilled diethyl ether. The resulting solution was then applied to a pre-coated, 0.25 mm silica gel TLC plate, with a UV_{254} indicator (Camlab) and run with ANALAR chloroform (BDH) and 5% ethanol. The plate was dried and the position of the sample was marked on the plate after examination under u.v. light (254 nm). The sample was then removed from the plate and the sterols were re-extracted from the silica powder using redistilled diethyl ether. The sterols contained within the sample were then identified using GC-MS; this method is described in full in Section 4.2.4.. A sample of authentic ergosterol (Aldrich) was separately processed in exactly the same way for comparison, taking care that no cross contamination of the samples could occur.

4.2.3 Sterol and ecdysteroid extraction from insect tissue.

Due to the quantity of cecid tissue needed to provide adequate amounts of sterol and ecdysteroid for analysis, it was decided to develop a method which could be used to obtain both compounds from a single tissue sample. It was also decided that paedogenetic larvae from the culture of *H. pygmaea* would be used as, in this species, the larvae tend to be found in densely packed groups on the agar plate (see Section 2.3.3) and as such are easier to collect than those of *M. speyeri.*

The *H. pygmaea* larvae were cultured as described in Section 2.2.1 and were collected individually from the agar plates; 0.8 g of paedogenetic *H. pygmaea* larvae were collected for use in this experiment. The insects were macerated in approximately 10 ml of a mixture of HPLC grade methanol (Sigma) and distilled water (70%/30%).

The homogenate was then centrifuged twice. Each time the supernatant was removed and fresh 70 % methanol was added. The pellet was then re-macerated twice, each time with 10 ml of 100% methanol, centrifuged, and the supernatants were combined with the others. The pellet was then re-extracted with HPLC grade acetone (Sigma) in order to remove any sterols present, centrifuged and the supernatant reserved.

The methanol/water fraction was then twice partitioned with HPLC grade hexane (Sigma). The hexane fractions were back extracted with 70%/30% methanol/water which was added to the original methanol/water fraction. The hexane fractions contained the sterols; they were combined with the acetone fraction which was obtained previously and evaporated to dryness under nitrogen at around 40 °C. GC-MS was then used to identify any sterols present, (see Section 4.2.4 for details of the method used).

The methanol/water fraction which contained the ecdysteroids was dried under nitrogen at around 40 - 50 ^{o}C. The sample was weighed and redissolved in a small volume of 10% methanol in water (5 mg in 1 ml) in order for it to be prepared for HPLC using C 18 Sep-Pak cartridges (Waters Chromatography).

The cartridges were activated with 5 ml methanol and 10 ml water. The sample was loaded onto the cartridge 1 ml at a time, then sequentially eluted with 2 ml of 10% methanol, 4 ml of 30% methanol in water. This 30% fraction contained the polar ecdysteroids. The cartridges were then eluted using 6 ml of 60% methanol in water, to elute the free ecdysteroids. Finally they were eluted with 8 ml of methanol. This elutes any apolar materials (e.g. fatty acyl esters).

All fractions were evaporated to dryness under nitrogen; the free ecdysteroids contained within the 60% methanol in water fraction were identified using HPLC-RIA (this is described in Section 4.2.5).

4.2.4 Identification of sterols using GC-MS.

The sterols obtained from both the fungal mycelium and the insect tissue (Sections 4.2.2. and 4.2.3. were identified using gas chromatography and mass spectrophotometry (GM-MS). This was done by me in the Department of Biochemistry, University of Liverpool, using facilities generously provided by Professor Huw Rees, to whom I am grateful.

Before analysis both samples were derivatised using N,O,-bis(triethylsilyl)trifluoroacetamide with 1% trimethyldiborosilane (BSTFA) (Pierce) and pyridine 1:1 v/v (approximately 10 µl of each) on a steam bath for 10 minutes.

Both were injected onto a Hewlett-Packard 5890 Series 1 gas chromatograph; column 25 metres H-P 1 (Hewlett-Packard), 0.2 mm i.d., 0.33 µm film thickness, helium

carrier gas, 10 psi. The temperature programme used was 50 °C - 150 °C at 25 °C per minute, then at 6 °C per minute to 280 °C. Sterols were detected using a HP 5970 mass selective detector (MSD). The mass range was *m/z* 50 - 550 and the scan time was 1 second.

The sterol sample from the insect tissue was very small; the identification for these sterols was confirmed by injecting a replicate sample onto a H-P 5890 Series II gas chromatograph coupled to a VG Quattro quadrapole mass spectrometer; column 12 metres (SGE) BP-1, 0.22 mm i.d., 0.1 μm film thickness, on column injection, helium carrier gas, 15 psi. The temperature programme used was 50 °C to 150 °C at 25 °C per minute and then 150 °C to 280 °C at 6 °C per minute. The mass range was *m/z* 40 - 500 with a scan time of 2 seconds.

4.2.5 Identification of ecdysteroid from insect tissue using HPLC-RIA.

This was also done by me in Professor Rees' lab at Liverpool. The free ecdysteroid fraction obtained from the insect tissue using the method described in Section 4.2.3 was loaded onto a Waters HPLC system consisting of: two model 6000A delivery pumps, with a U6K injector, a M680 gradient controller, a M441 absorbance detector and a reversed-phase system Nova-Pak C_{18} Radial Pak Cartridge (10 cm × 8 mm i.d.; 4μ.; 60 Å; Waters).

A linear solvent gradient of 40 - 60% (v/v) methanol (BDH) in water over 40 minutes was used at a flow rate of 1 ml per minute. The absorbance was set at 254 nm. All HPLC solvents were filtered through a 47 mm Nylaflo nylon membrane (0.45 μm pore

size) (Gelman Sciences, Michigan, U.S.A) and degassed prior to use. The sample was collected as 0.5 ml fractions on a FRAC 100 fraction collector (Pharmacia) and dried using centrifugal evaporation (GYROVAP, Uniscience, U.K.) and analysed as described below.

Retention times for ecdysone, 20-hydroxyecdysone and makisterone A standards were estimated by running 10 µl of standard solutions (each stock solution contained 1µg per ml) on the above system. All three standards were purchased from Simes, Milan, Italy.

The radioimmunoassay performed uses ecdysone as a standard competing for binding to the H-22 antiserum with [23, 24 (n) - ^{3}H] ecdysone. Bound and unbound ecdysteroid were separated by ammonium sulphate precipitation (Mendis, Rose, Rees and Goodwin 1983). The H-22 antiserum was raised against an ecdysone 22-hemisuccinate conjugate, and therefore its specificity is directed toward the steroid nucleus.

Ecdysone standards of the following concentrations 0, 20, 50, 100, 200, 500, 1000 and 2000 pg per tube were prepared in duplicate by dilution to 50 µl in borate buffer with 5% inactivated rabbit serum (I.R.S.)(Sigma). Ten µl H22 antisera was diluted to 11 ml with borate buffer containing 5% I.R.S. Radiolabelled ecdysone was diluted with borate buffer with 5% I.R.S. in order to give 8000 c.p.m. per assay tube. Assay solutions were added to the ecdysone standards, the samples and tubes to show total counts and non-specific binding, in the following way:

	N°. of Tubes	Borate Buffer	Antiserum	Label
Samples	80	50 µl	100 µl	50 µl
Standards	16	50 µl	100 µl	50 µl
Total Counts	2	50 µl	_	50 µl
Non-specific binding	2	150 µl	_	50 µl

Table 4.1 Ecdysteroid RIA

After 18 hours incubation at 4 °C 200 µl of borate buffer saturated with ammonium sulphate was added to all tubes except the 'totals'. The tubes were then incubated for 20 minutes at 4 °C, spun at 12 000 r.p.m. for 15 minutes and the supernatant was removed. The pellet was then resuspended in 500 µl borate buffer, 50% saturated with ammonium sulphate, incubated for a further 20 minutes at 4 °Ç before being centrifuged and having the supernatant removed. The pellet was then redissolved in 100 µl of distilled water and 1 ml of 'Starscint' (Packard) scintillation fluid before being counted on an LKB Wallac, 1219 RackBeta liquid scintillation counter. The details of all solutions used can be found in Appendix 5.

4.3 Results.

4.3.1 Treatment of cecids with hormone analogues.

To test whether growth and paedogenetic reproduction in *M. speyeri* larvae could be affected by the application of hormones and their analogues, experiments were carried out to monitor the effects of various analogues on the ability to produce paedogenetic offspring. The results are shown in Tables 4.2 - 4.5. In each experiment, since there were no significant differences between either of the parameters measured in successive generations of insects in the same treatment category, the results for successive generations of the same category were pooled.

Paedogenetic larvae from the stock culture of *M. speyeri* were placed onto plates of 2% malt-extract agar containing increasing concentrations of an ecdysteroid analogue (RH 5849 or RH 5992). The numbers of insects produced per generation and the time taken for each generation to be produced are shown in Table 4.2. The results in this table demonstrate that there was a statistical difference in the paedogenetic development and reproduction of larvae treated with an ecdysteroid analogue. However, on comparison of the mean values using Newman-Keuls analysis, it became clear that there was no significant difference between the control treatments in both sets of insects and those treated with the highest dose of analogue; therefore no further comparison of means was performed.

From the data it is clear that although the insects in the different treatments did produce varying numbers of offspring this variation was not significantly different for the control insects from those treated with the highest dose of either RH 5849 or RH

5992. The insects from all treatments were able to produce offspring paedogenetically which in turn went on to produce offspring. The time taken for the larvae to produce the next generation differed slightly but this was no more varied than the insects kept in stock culture.

M. speyeri larvae were then treated directly with pyriproxyfen, a JH analogue, in order to monitor the effects of this chemical on paedogenetic reproduction. The numbers of insects produced per generation and the time taken for each generation to be produced are shown in Table 4.3. The results in this table show that there was no significant difference ($p = 0.209$) in the time taken for pyriproxyfen-treated *M. speyeri* larvae to produce paedogenetic offspring. A significant difference ($p = 0.000009$) was found between the numbers of offspring produced by the larvae which had been treated with pyriproxyfen and the control insects. When the means were compared using Newman-Keuls analysis it was found that the insects that had been treated with 30 ppm pyriproxyfen produced significantly ($p = 0.00013$) fewer larvae per generation than those in the control treatment. There was no significant difference between those insects which had been treated with lower doses and the control larvae.

During the experiment the effect that was seen was a reduction in the fitness of the insects which had been treated with the solution containing 30 ppm pyriproxyfen. They produced fewer offspring and of those that were produced some were found to be still born. Many of the larvae which had been treated with 30 ppm of pyriproxyfen did not produce any offspring at all, however, they did not appear to be affected in any way other than this. This observation would support the findings of Went (1978b) who

found that during *in vitro* culture of *H. pygmaea* ovaries JH treatment inhibited follicle formation.

The above experiment was repeated again using direct treatment of *M. speyeri* larvae with pyriproxyfen. The numbers of insects produced per generation and the time taken for each generation to be produced are shown in Table 4.4. The results in Table 4.4 show that no significant difference was found between insects which had been treated with pyriproxyfen and control insects. The effects seen on the insects initially dosed with pyriproxyfen were not seen during this experiment. In order to clarify the findings of the first experiment further studies were carried out to monitor the effects of another JH analogue, fenoxycarb, and JH III on the paedogenetic growth and development of *M. speyeri* larvae.

Differing concentrations of fenoxycarb and JH III were included in the media on which the insects were living. This method was used in order to provide a constant dose of exogenous JH in an attempt to eliminate any influence that the timing of application may have on the results of the experiment. The numbers of insects produced per generation and the time taken for each generation to be produced is shown in Table 4.5 The results in Table 4.5 show that treating *M. speyeri* larvae with either fenoxycarb or JH III gave no significant difference in either the number of offspring produced by the larvae or the time taken for each generation to be produced. These experiments were carried out on a number of occasions but on each occasion similar results were obtained.

4.3.2 Identification of dietary sterol from GC-MS.

The sterols obtained from the fungal mycelium, described in Section 4.2.2, were analysed using GC-MS. The total ion chromatogram, seen in Figure 4.2 shows 4 discernible peaks. The peak which can be seen to the right of peak 4 (Figure 4.2) represents the unsilylated sterols present in the sample. Of these 4 peaks, peak 1 contained the majority (80%) of the total sterols detected.

The sterols represented by the 4 peaks were identified by comparing their spectra and retention times on the column with those of authentic standards supplied originally by the M.R.C. Sterol Reference Collection (Queen Mary and Westfield College, University of London)(Girling 1991). Both methods of identification were used because, although Δ^8 and Δ^7-sterols have similar spectra, they can be distinguished by the comparison of their retention times as Δ^8- sterols are much earlier than the corresponding Δ^7-sterols.

Figures 4.3 - Figure 4.6 show the mass spectra for the four peaks. The ions seen in each sterol were identified according to the observed mass charge (*m/z*) and the relative intensity. The data for each spectrum is shown in table 4.6. Using the data shown in Table 4.6 and the retention times of the four sterols they were identified as follows:

Peak 1 Ergosta-5, 7, 22-trien-3β-ol TMS ether (Ergosterol TMS).

Peak 2 Ergosta-7, 22-dien-3β-ol TMS ether.

Peak 3 Ergosta-5, 7-dien-3β-ol TMS ether.

Peak 4 Ergosta-7-en-3β-ol TMS ether.

4.3.3 Identification of insect sterols from GC-MS.

The sterols obtained in the hexane fraction from the insect tissue (Section 4.2.3) were analysed directly, without any further preparation. This was due to the fact that preliminary work had shown that the samples contained very small amounts of sterols. Figure 4.7 shows that there were no distinct peaks visible in the region expected for sterols on the total ion chromatogram of the TMS-ether derivatives.

On further examination the spectra for two sterols were discernible, (Peak 1, $[M]^+$ 454; Peak 2, $[M]^+$ 468). Figure 4.8 shows how reconstructed ion currents for these ions revealed two peaks (Peak 1, *m/z* 454, R_T [retention time relative to cholesterol TMS] was approximately 1.02; Peak 2, *m/z* 468, R_T 1.08; c.f. R_T of authentic ergosterol TMS, 1.07 and campesterol TMS, 1.09).

The mass spectrum for peak 2 (Figure 4.9) was similar to that observed for ergosterol TMS (obtained from the fungal mycelium, Section 4.3.2.); the spectrum for the ergosterol obtained from the insect tissue was much weaker as the sample was much smaller. Similarly peak 1, the other sterol isolated from the insect tissue, shown in Figure 4.10 gave a weak spectrum with appreciable background. Ions were observed at *m/z* (relative intensity) shown in table 4.7.

The spectra shown in Table 4.7 indicates a C_{27} sterol triene with two ring double bonds and one side chain double bond. The prominent ion at *m/z* 323 is characteristic of the TMS derivative of a $\Delta^{5,7}$ - sterol corresponding to $[M-131]^+$ and arises by the loss of a

C - 1 to C - 3 fragment together with 2 further hydrogens (Brooks, Horning and Young 1968, Goad 1991). Other diagnostic fragment ions for $\Delta^{5,7}$ - sterols were present but were somewhat obscured by high background values of around *m/z* 160.

The ions present at *m/z* 253 and 251 probably represent the loss of the side chain by the cleavage of the C - 17/ C - 20 bond and are generally observed in sterols bearing a Δ^{22} or Δ^{24} bond (Wyllie and Djerassi 1968, Goad 1991). However the absence of a major ion in the spectra at *m/z* 69, which is observed for Δ^{24} sterols, which arises by the cleavage of the allylic C - 22 to C - 23 bond, may suggest that peak 1 represents a Δ^{22} sterol. This theory is supported by the retention time, since a Δ^{24} bond may result in a longer retention time than that seen for peak 1. The combined evidence suggests that peak 1 is cholesta - 5, 7, 22- 3 β - ol, although the evidence is based primarily on the mass spectrum of minute amount of unpurified sterol fraction.

4.3.4 Identification of ecdysteroid from insect tissue.

In order to ensure that no contamination had been introduced to the HPLC system a solvent blank was injected onto the system and assayed in the same way as the ecdysteroid sample. The fractions collected from this blank run showed that no immunoreactive compounds were present in the system. The standard hormone solutions which had been injected onto the column gave the following retention times for comparison with any immunoreactivity found in the cecid ecdysteroid sample: 20-hydroxyecdysone 13.3 minutes, makisterone A 19.4 minutes and ecdysone 22 minutes.

The fractions obtained from the cecid ecdysteroid sample were analysed using the radioimmunoassay described in Section 4.2.5. The fractions from the HPLC were measured for cross reactivity with the H22 antibody. Some fractions which showed immunoreactivity can be attributed to non-specific binding of contaminants within the assay, others may be due to ecdysone-like immunoreactivity. Figure 4.11 shows that the sample contained considerable amounts of material absorbing at 254 nm which may have interfered with the separation. Thus, it is possible that ecdysteroids in the sample may not have eluted exactly at the times indicated by the standards. RIA of the eluted fractions revealed that the amounts of ecdysteroids present in the sample were relatively small in relation to the detection limit of the assay (~ 20 pg). Figure 4.12 shows only those fractions which exceeded the detection limit.

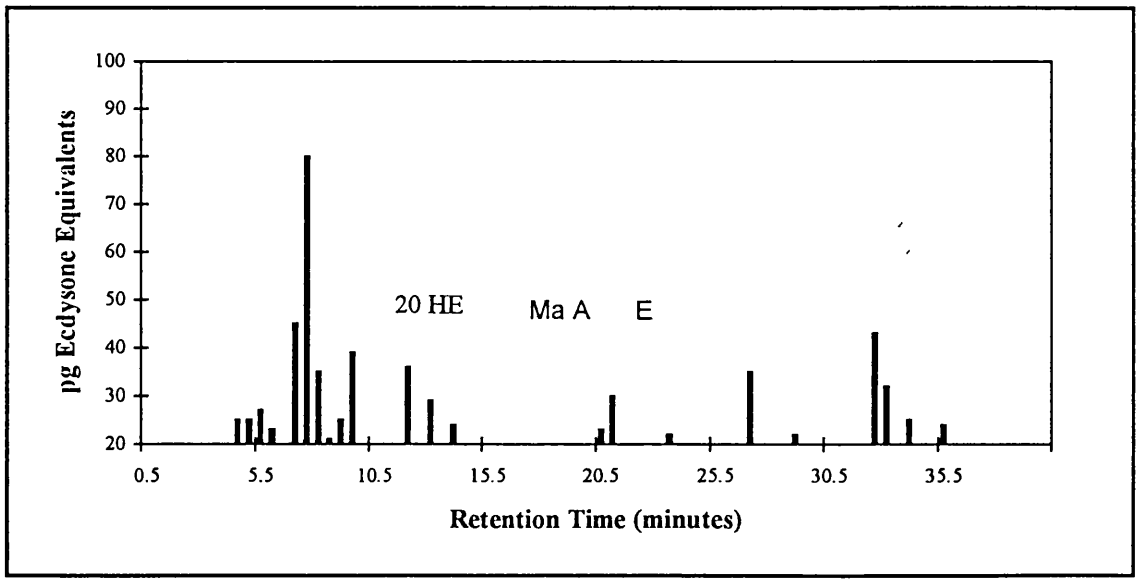


Figure 4.12. Graph showing fractions from cecid ecdysteroid sample exhibiting ecdysone-like immunoreactivity in terms of pg of ecdysone equivalents. The retention times of three insect hormone standards are marked 20-hydroxyecdysone (20HE), makisterone A (Ma A) and ecdysone (E).

The HPLC profile (Figure 4.11) shows that one or more peaks of immunoreactivity that elute early (4 - 9 minutes); these are more polar than 20-hydroxyecdysone. They are likely to represent polar metabolites or conjugates. Immunoreactivity eluting in late fractions (26 minutes and later) is likely to represent apolar metabolites (Rees and Isaac 1985). Evidence for free ecdysteroids was not conclusive, but some tentative deductions can be made. It can be seen from Figure 4.12 that no ecdysone-like immunoreactivity was seen in the fractions which shared a retention time with the makisterone A standard solution, suggesting that makisterone A is absent. There is some evidence to suggest that there was a peak of ecdysone-like immunoreactivity in the fractions collected between 12 and 14 minutes, which corresponds with the retention time of the standard solution of 20-hydroxyecdysone. It can be seen however that no immunoreactivity can be seen in the fractions which co-elute with ecdysone (at 22 minutes). The identity of the peak at around 25 minutes is unknown.

Tables 4.2 - 4.5 show the results obtained when *M. speyeri* larvae were treated with hormone analogues. The results are described in terms of the numbers of offspring produced by each individual and the time taken for each generation to be produced. All numbers given in these tables represent the mean value for each treatment (± standard deviation from the mean) and n represents the number of replicate Petri plates.

Treatment	RH 5992			RH 5849		
Conc. (ppm)	**time**	**number**	**n**	**time**	**number**	**n**
30	4.0 (0.0)	28.3 (3.5)	4	4.0 (0.1)	24.4 (0.8)	5
10	4.9 (0.6)	14.9 (10.9)	8	4.3 (0.3)	17.2 (2.5)	5
3	4.0 (0.0)	24.6 (1.5)	5	4.0 (0.0)	24.5 (11.5)	4
1	5.4 (0.54)	13.0 (13.9)	5	5.0 (0.8)	17.3 (1.2)	6
0	4.6 (0.74)	24.4 (5.2)	8	4.6 (0.2)	24.3 (3.7)	8
	f= 5.51	f= 3.29		f = 3.3	f = 1.39	
	p= 0.003***	p = 0.02***		p = 0.02***	p = 0.266 NS	

Table 4.2 The effects of RH 5849 and RH 5992 on paedogenetic *M. speyeri* larvae.

Pyriproxyfen Conc. (ppm)	Time Taken	Number produced	n
30	7.8 (0.63)	5.2 (7.0)	10
10	6.0 (1.49)	11.7 (10.5)	10
3	5.6 (1.17)	27.8 (4.56)	10
1	5.6 (1.42)	10.5 (10.23)	10
0	4.7 (0.82)	15.5 (10.04)	10
	f = 9.72	f = 1.52	
	$p = 0.209^{NS}$	p = 0.000***	

Table 4.3 The effects of a JH analogue, pyriproxyfen, on paedogenetic *M. speyeri* larvae.

Pyriproxyfen Conc. (ppm)	Time Taken	Number produced	n
30	7.0 (0.53)	19.1 (6.58)	15
10	7.13 (0.63)	19.8 (7.26)	15
3	6.8 (1.01)	19.2 (7.36)	15
1	7.06 (0.88)	16.3 (6.05)	15
0	6.3 (0.89)	19.8 (7.29)	15
	$f = 2.36$	$f = 0.69$	
	$p = 0.061^{NS}$	$p = 0.603^{NS}$	

Table 4.4 The effects of a JH analogue, pyriproxyfen, on paedogenetic *M. speyeri* larvae.

Treatment	Fenoxycarb			JH III		
Conc. (ppm)	time	number	n	time	number	n
30	6.53 (0.51)	19.7 (7.3)	15	5.6 (0.51)	21.4 (8.05)	10
10	6.46 (0.5)	18.9 (7.9)	15	5.3 (0.48)	15.5 (5.02)	10
3	6.78 (0.57)	22.5 (5.5)	15	5.6 (0.69)	14.4 (8.4)	10
1	6.81(0.54)	21.5 (6.4)	15	5.6 (0.84)	15.9 (9.24)	10
0	6.73 (0.7)	21.6 (5.8)	15	5.3 (0.48)	21.4 (5.25)	10
	$f = 1.11$	$f = 0.9$		$f = 0.69$	$f = 1.67$	
	$p = 0.35^{NS}$	$p = 0.46^{NS}$		$p = 0.59^{NS}$	$p = 0.17^{NS}$	

Table 4.5 The effects of fenoxycarb and JH III on paedogenetic *M .speyeri* larvae.

	Peak 1	Peak 2	Peak 3	Peak 4
Ion	***m/z* (%)**	***m/z* (%)**	***m/z* (%)**	***m/z* (%)**
M^+	468 (21%)	470 (34%)	470 (16%)	472 (80%)
-Me	453 (2%)	455 (21%)	455 (3%)	457 (22%)
-TMSiOH	378 (18%)	380 (7%)	380 (23%)	382 (7%)
-TMSiOH-Me	363 (100%)	365 (6%)	365 (100%)	367 (22%)
-TMSiOC$_3$H$_6$	377 (4%)		339 (85%)	
-C$_9$H$_{17}$		345 (30%)		
-C$_9$H$_{19}$				345 (12%)
-C$_9$H$_{17}$-2H		343 (94%)		
-TMSiOH-C$_9$H$_{17}$	253 (26%)	255 (100%)		
-TMSiOH-C$_9$H$_{19}$			253 (14%)	255 (100%)
-TMSiOH-C$_9$H$_{17}$-2H	251 (9%)	253 (17%)		

Table 4.6 Mass spectral data for the 4 sterols isolated from a sample of the fungus *C. purpureum* showing mass charge and relative intensity.

Ions present in peak 1	*m/z* (% relative to total)
$[M]^+$	454 (19.6 %)
$[M - CH_3]^+$	439 (5.6 %)
[M - TMS]+	364 (18.69 %)
$[M - TMS - CH_3]^+$	349 (100%)
$[M - side\ chain - 2H]^+$	341 (27.1 %)
$[M - (C\text{-}1\ to\ C\text{-}3) - TMS]^+$	323 (61.7 %)
$[M - S.\ C. - TMS]^+$	252 (10.28 %)

Table 4.7 Mass spectral data for Sterol 1 extracted from *H. pygmaea* larvae.

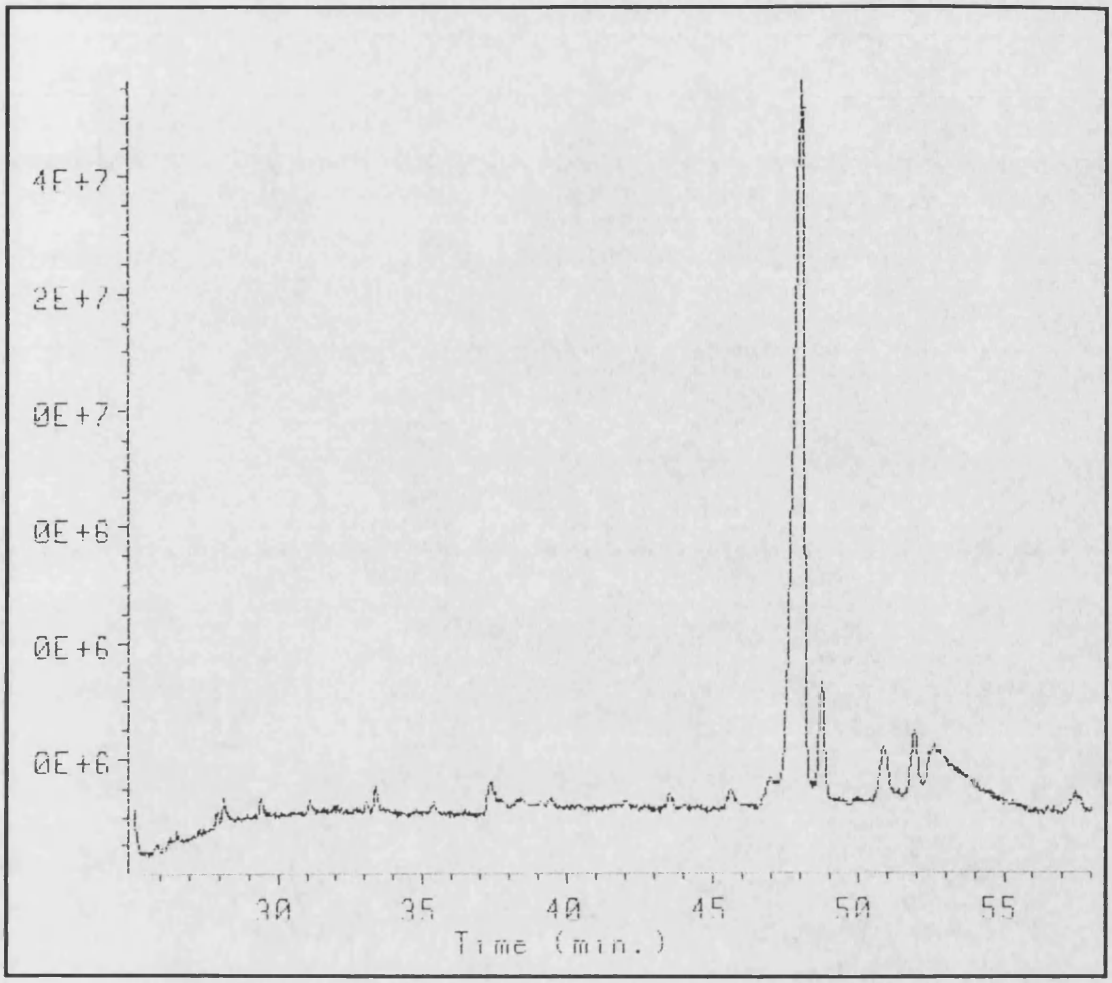


Figure 4.2 Total Ion Current for sterol sample extracted from *C. purpureum* mycelium.

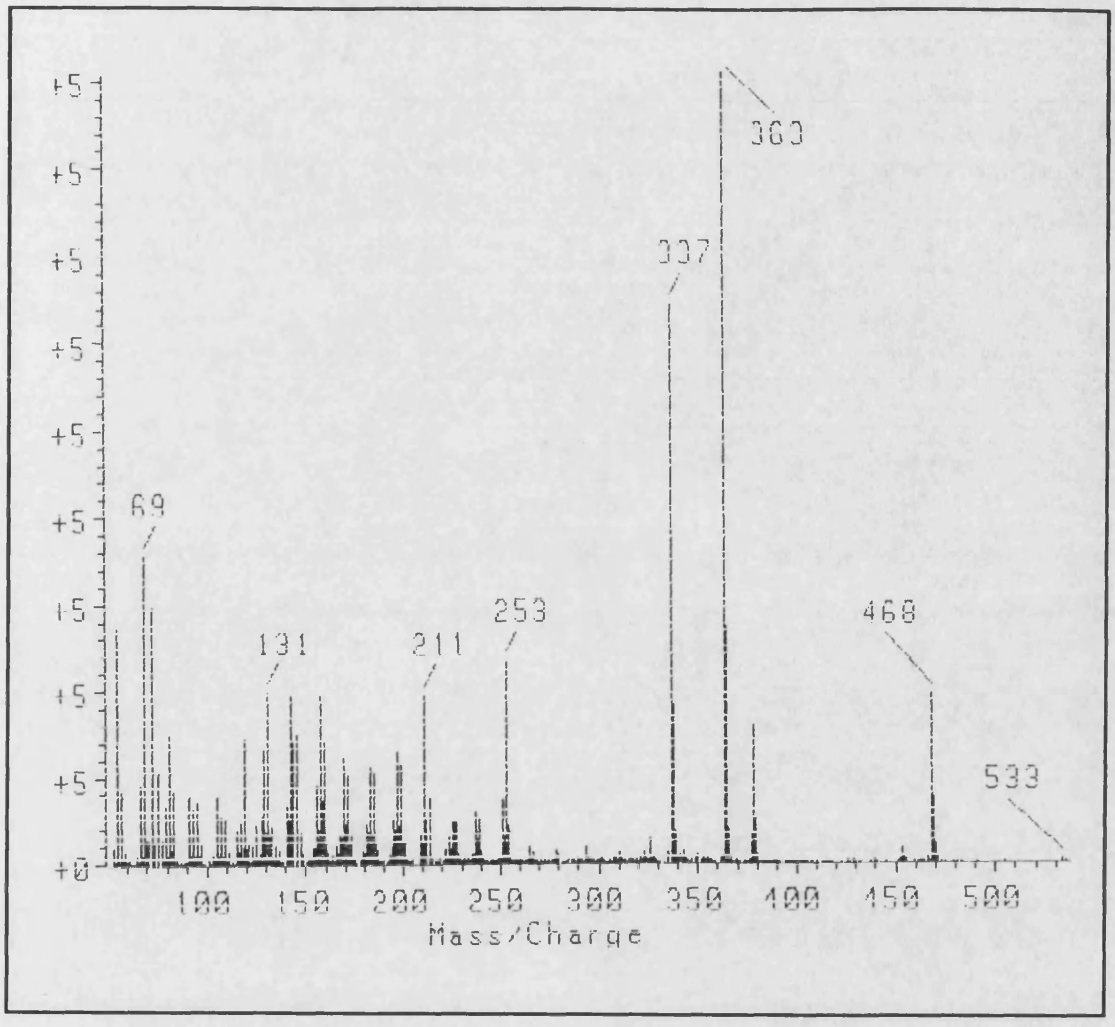


Figure 4.3 Mass spectrum for peak 1; ergosta-5, 7, 22-trien-3β-ol TMS ether (ergosterol TMS)

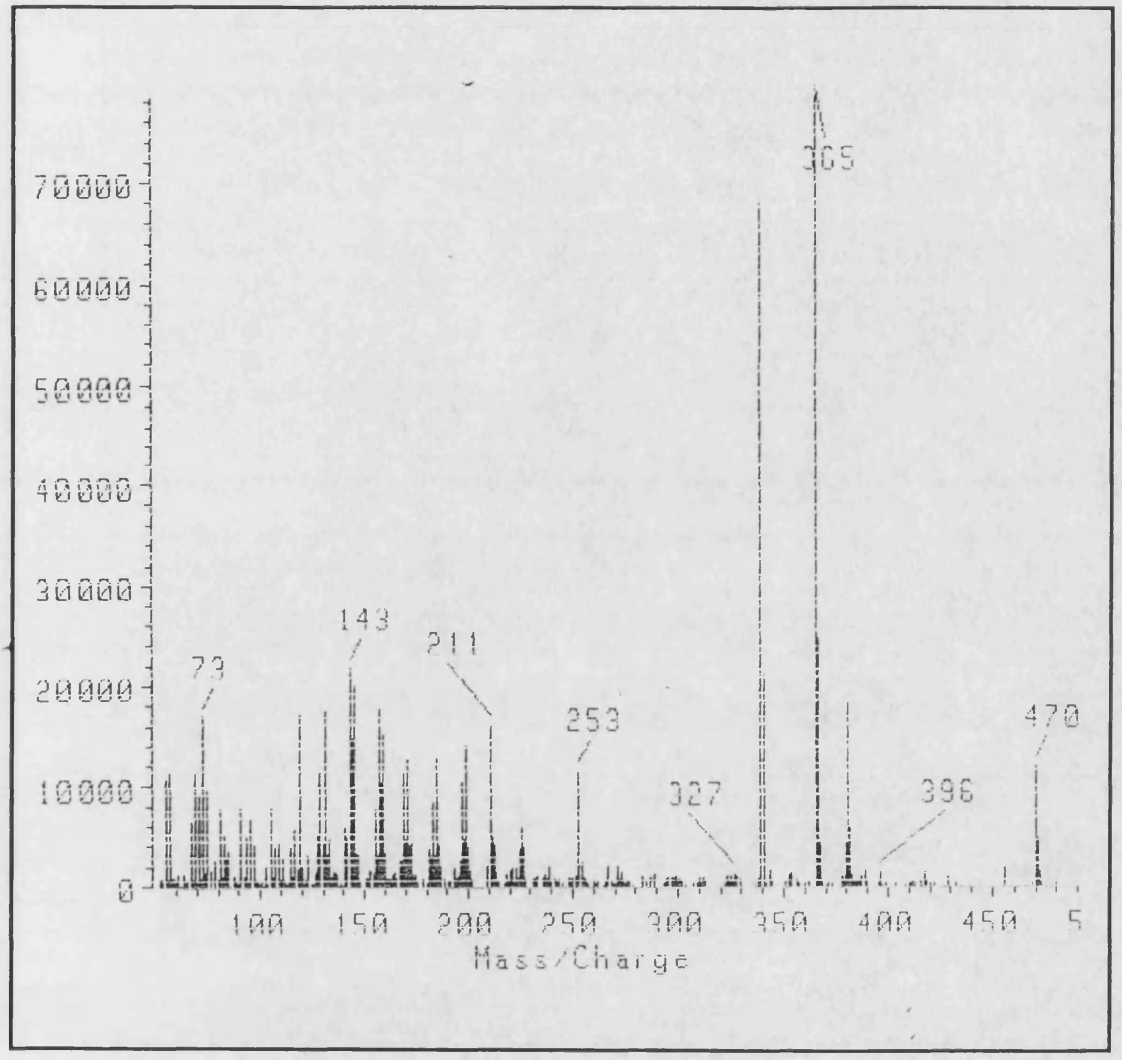


Figure 4.4. Mass spectrum for peak 2; ergosta-7, 22-dien-3β-ol TMS ether.

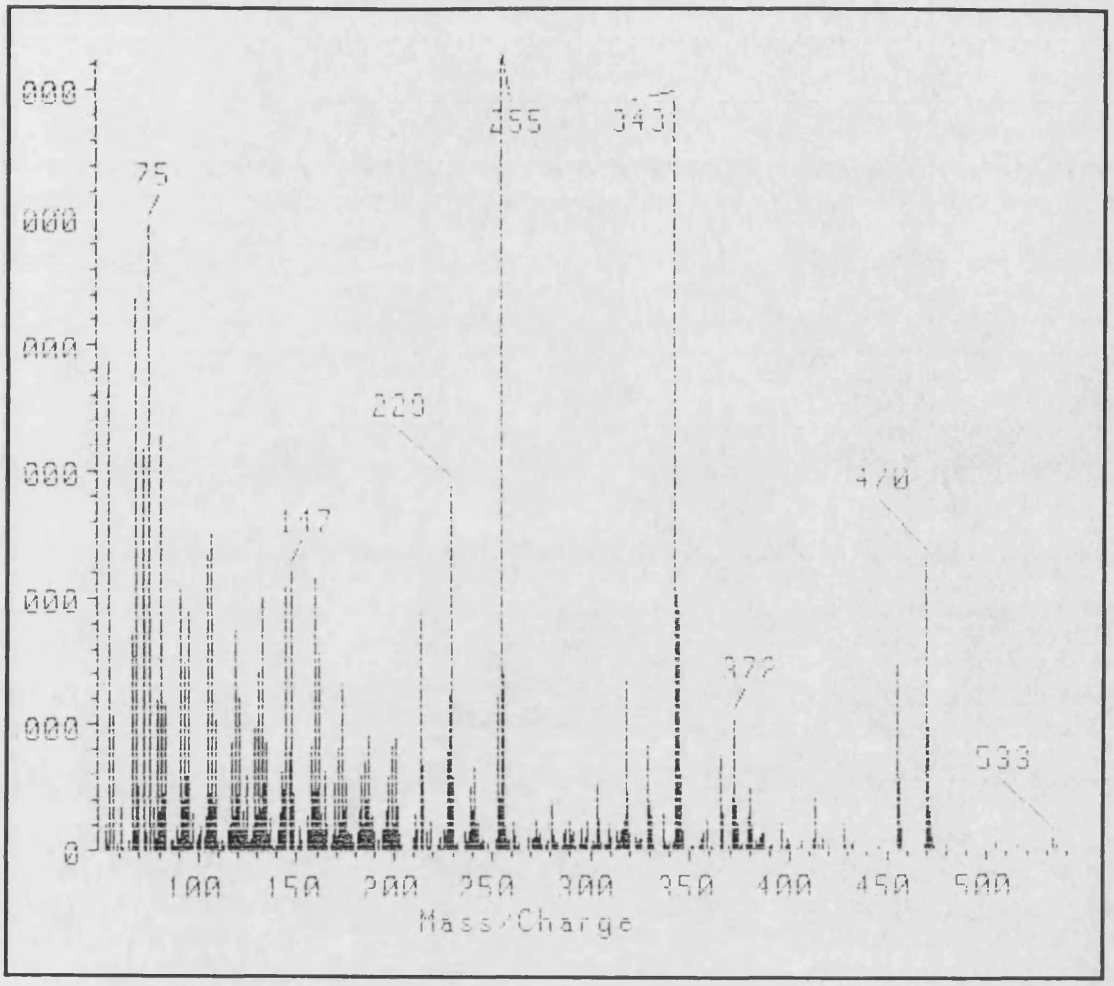


Figure 4.5 Mass spectrum for peak 3; ergosta-5, 7-dien-3β-ol TMS ether.

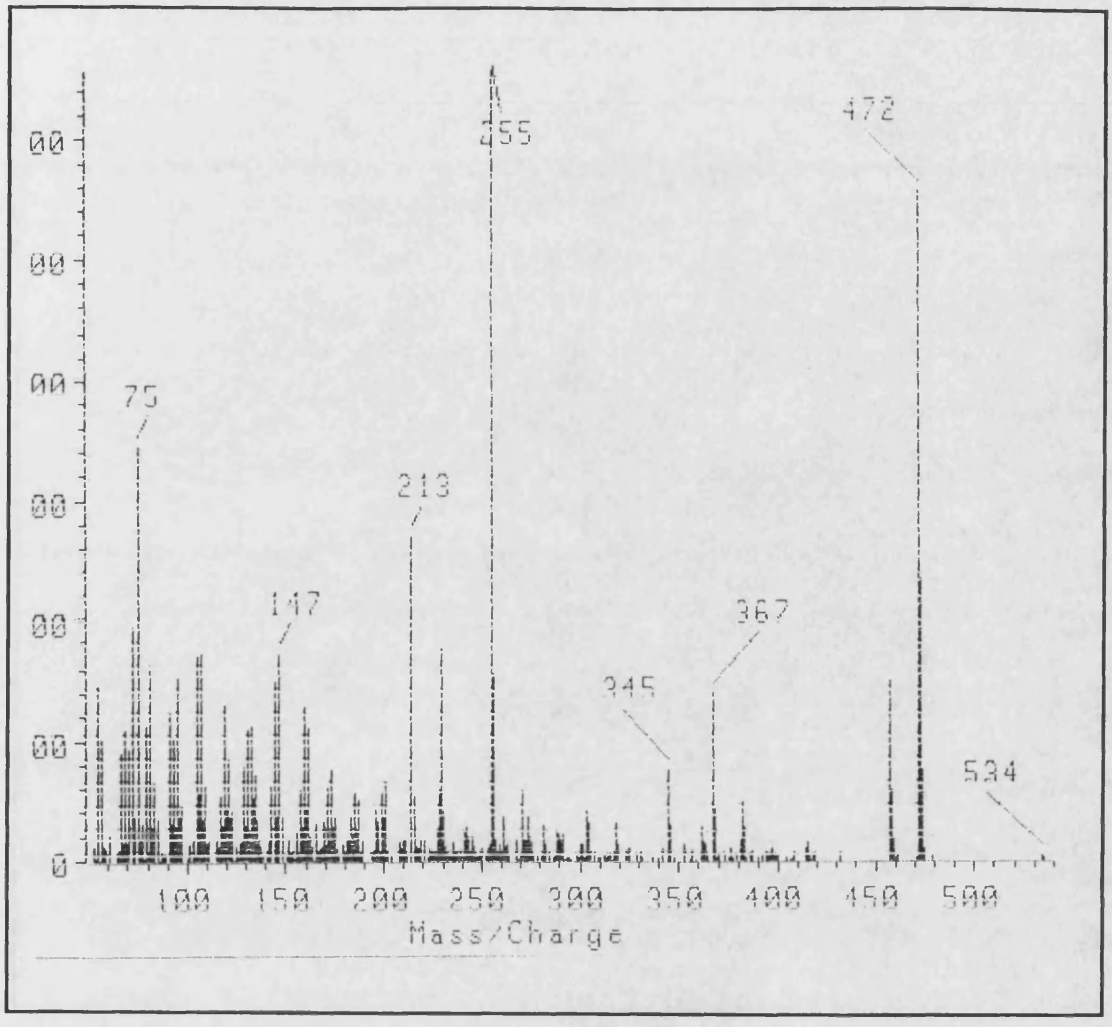


Figure 4.6 Mass spectrum for peak 4; ergosta-7-en-3β-ol TMS ether.

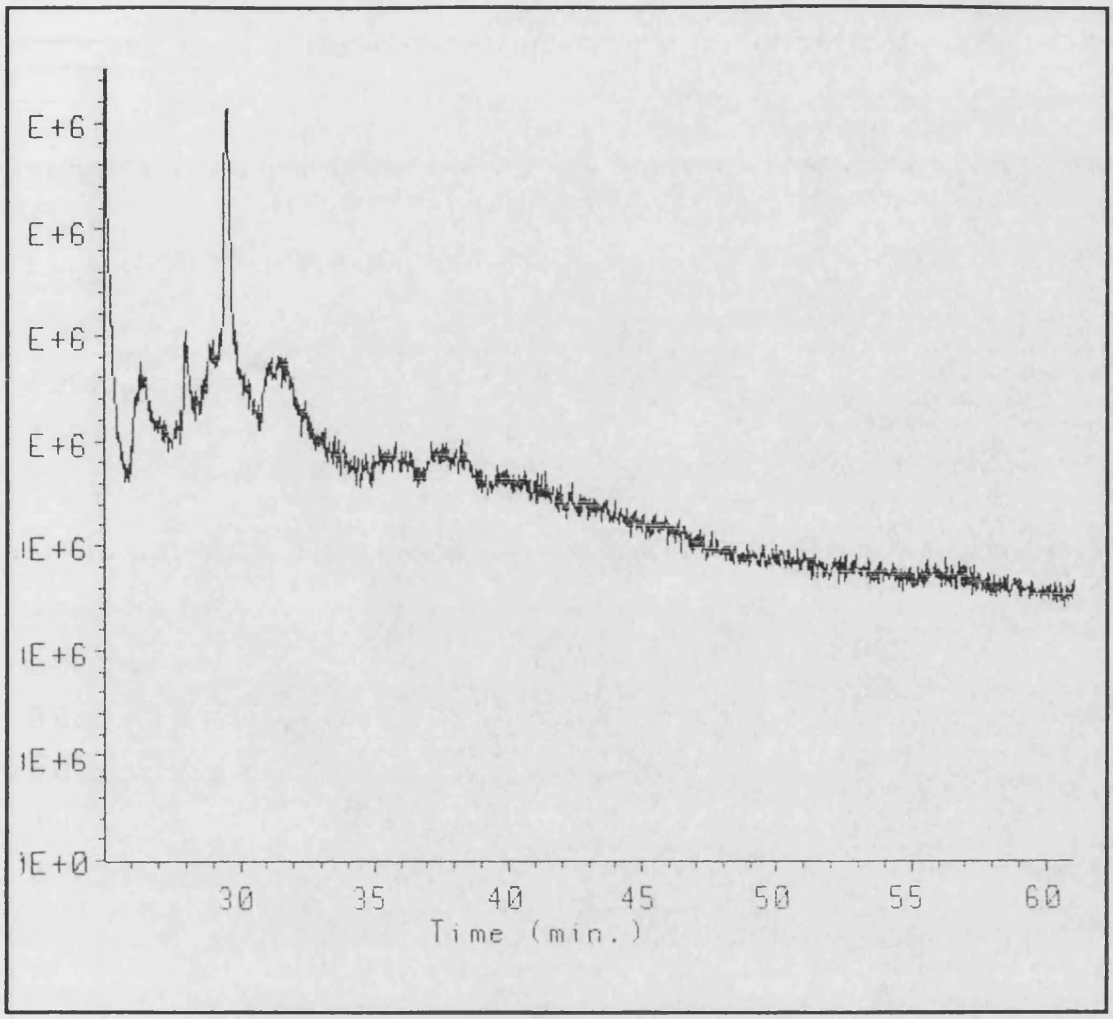


Figure 4.7 Total Ion Current for *H. pygmaea* sterol sample.

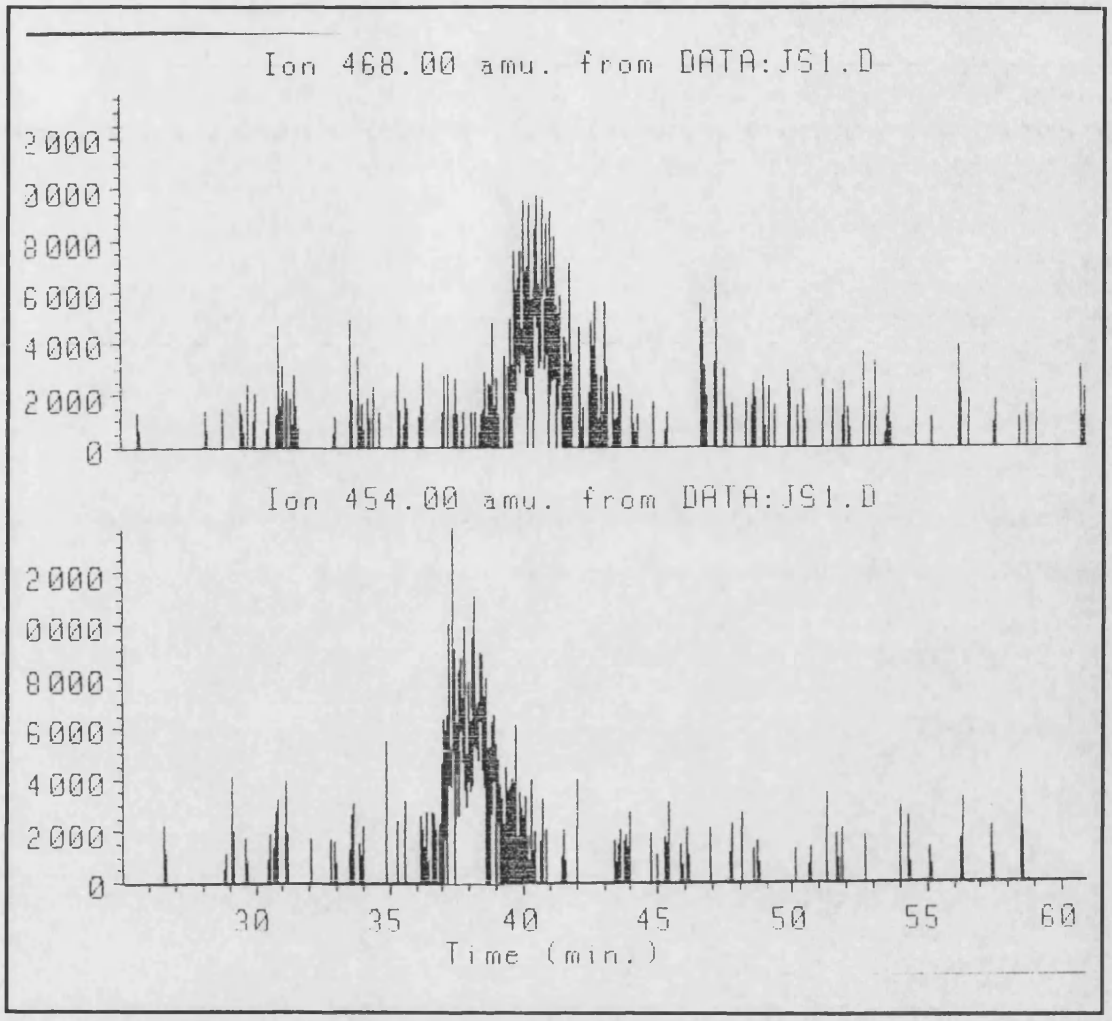


Figure 4.8 Reconstructed ion current for *H. pygmaea* sterol sample.

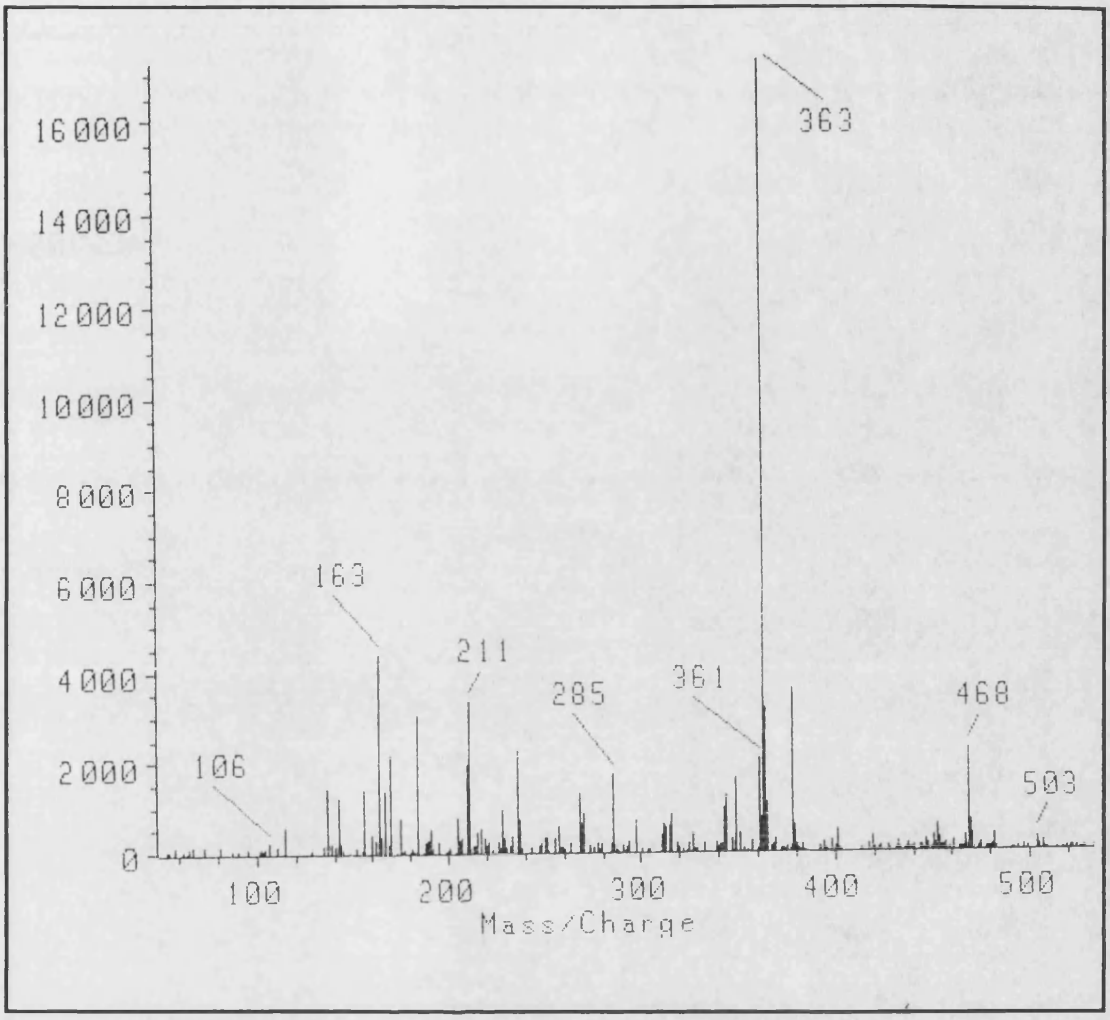


Figure 4.9 Mass spectrum for ergosterol from *H. pygmaea* sterol sample.

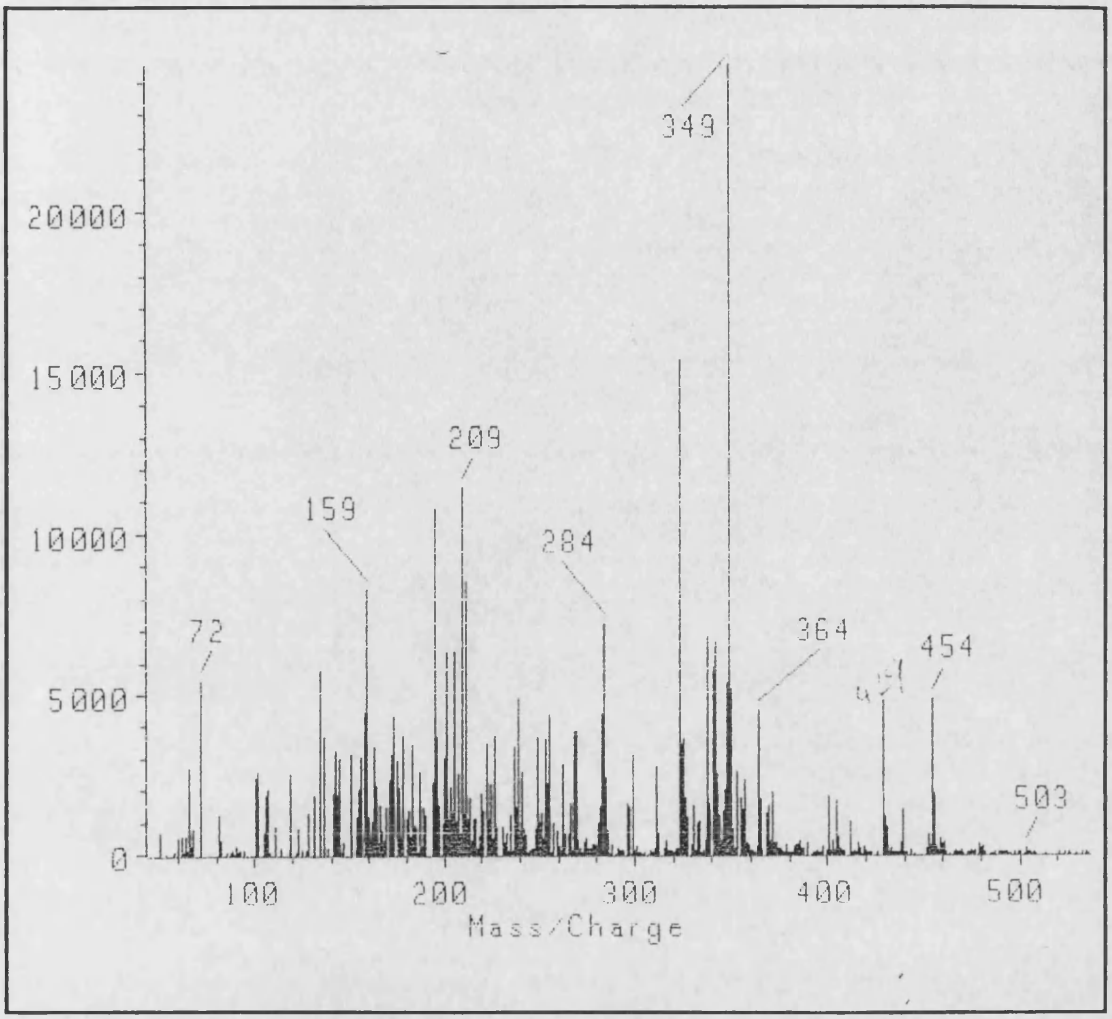


Figure 4.10 Mass spectrum for sterol from *H. pygmaea* sample; cholesta-5, 7, 22-3β-ol.

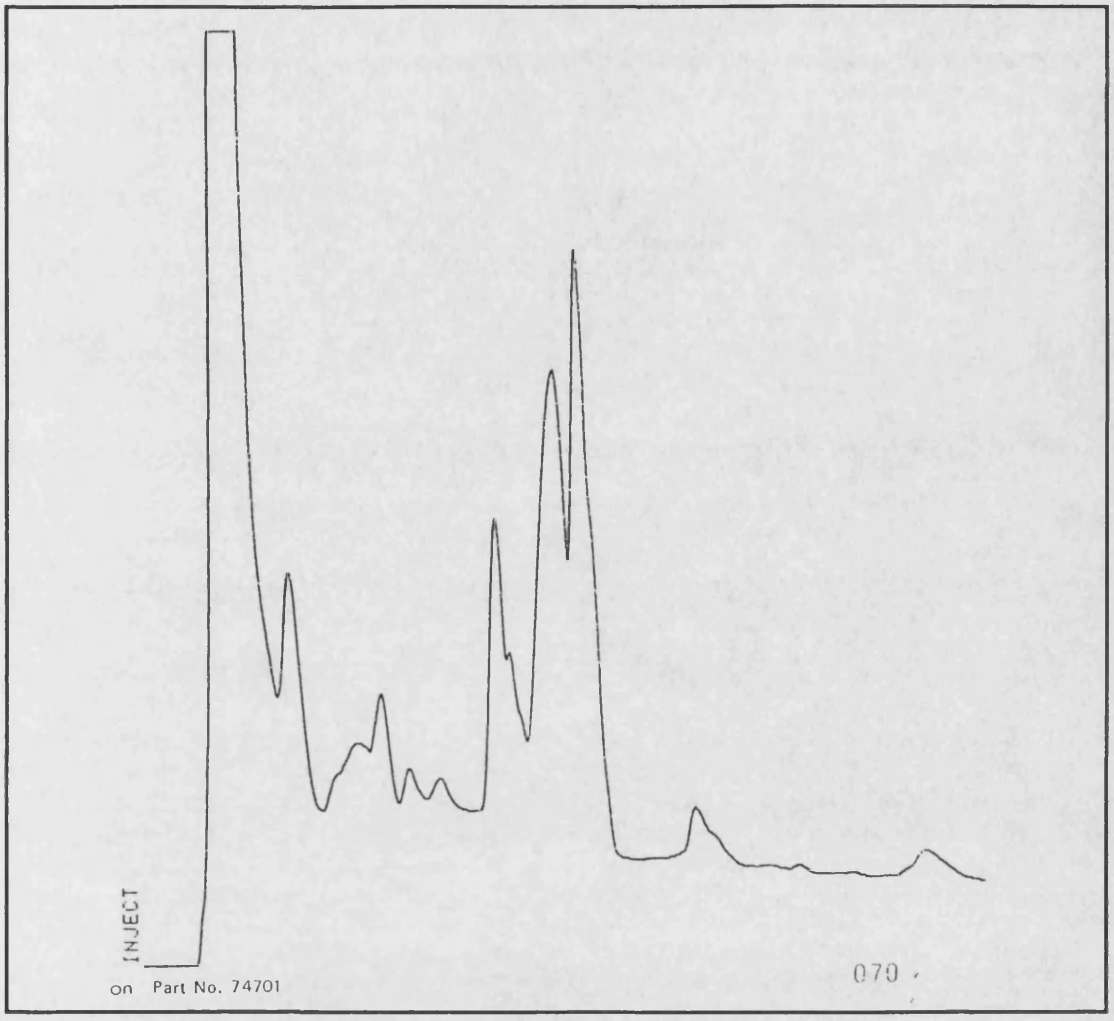


Figure 4.11HPLC trace for *H. pygmaea* ecdysteroid sample. Chart speed 0.5 cm/minute. Vertical scale = A254, 1.0 AUFS (absorbance units full scale).

4.4 Discussion.

No obvious effects were seen on development and reproduction of cecid larvae which had been treated with the 20-hydroxyecdysone analogues RH 5849 and RH 5992. There was no evidence of the larvae undergoing premature moults or experiencing any deleterious effects. Cecid larvae are thought to moult (Matuszewski 1982), but during the course of this study the process has rarely been observed as the shed cuticle is not readily visible.

When *M. speyeri* larvae were initially treated with the JHa pyriproxyfen, the larvae treated showed a significant reduction in the number of offspring that they produced. This result supports the findings of Went (1978b) who found that in *in vitro* culture the application of JH to the developing ovaries arrested follicle formation. These results proved to be difficult to replicate. When the same experiment was repeated, no difference was seen between the insects which had been placed in the analogue and the control insects which had been placed in acetone.

Another set of *M. speyeri* larvae were treated with another JHa, fenoxycarb, and JH III by incorporating them with the agar on which they were cultured. No effects were observed; all of the larvae in the experiment grew and developed normally and gave rise to further populations of paedogenetic offspring.

The difficulty experienced in replicating these results may have been due to the timing of the application, as sensitive periods are a very important factor in the response of insects to JH (Nijhout 1994). If this was the case it was assumed that the lack of

response observed in the second batch of insects treated with pyriproxyfen would not have been seen in the insects which were cultured on JH and JHa media as the exogenous hormone levels should have remained throughout the experiment.

The failure to repeat the initial results obtained was not due to a build up of resistance in the insect population. The insects used in the JH experiments were always from stock cultures and care was taken never to contaminate these cultures with JH.

JH is a very unstable hormone (Nijhout 1994) and is degraded by sunlight, esterases and being at room temperature when purified. Although this may explain the poor performance of the JH on the cecids it does not explain the poor reproducibility of the results obtained with the JHas which are produced as IGRs and are designed to be more robust in order for them to fulfil a function as IGRs for agricultural situations.

In the first pyriproxyfen experiment an effect was seen on the treated larvae. This was only seen at the highest concentration, 30 ppm pyriproxyfen. This is a very high dose; when the size of the cecid larvae is taken into account it is somewhat surprising that a response to the JHa was not seen at lower concentrations.

This apparently poor response to JHas by the *M. speyeri* larvae may be due to the resistant nature of their cuticle. Throughout the project difficulties have been experienced while fixing and preparing these insects for microscopy and other procedures. It may be the case that no effect from the hormone analogues was seen because the exogenous chemicals were unable to penetrate the cuticle. The poor

response of these insects to insect hormone analogues and the difficulty in reproducing any effects caused by such chemicals suggests that *M. speyeri* and other paedogenetic cecidomyiidae may be far from ideal candidates for control with hormonally acting IGRs.

Analysis of the fungal mycelium which is the sole source of dietary sterol for the mycetophagous cecids using GC-MS showed that ergosta- 5, 7, 22 - trien - 3β - ol (ergosterol) was the major sterol present in fungal mycelium with only very minor amounts of 3 other ergosterol derivatives, and that the mycelium did not contain any cholesterol. It may therefore be assumed that since the cecids had no access to a source of cholesterol or any C_{22} sterols that they must either dealkylate the ergosterol that was present in their diet to synthesise ecdysone and 20-hydroxyecdysone, or use alternative hormones.

Two sterols were found to be present in the insect tissue which was analysed using GC-MS. The first was ergosterol. This would have been present on the cuticle of the insect and in the gut contents. The second sterol found was tentatively identified as cholesta - 5, 7, 22 - 3β - ol. This steroid has not previously been identified in insects although it has been previously documented as occurring in the oyster, *Crassostrea virginica,* (Teshima and Patterson 1980) and in the Red Sea sponge, *Biemna fortis,* (Delseth, Kashman and Djerassi 1979).

Although the identification of the second sterol present in *H. pygmaea* was from a minute quantity taken from an unrefined sample it was clear from the results that it was

a C_{27} sterol; this clearly indicates that this cecid is able to dealkylate the dietary sterol. If this is the case it is possible that the developmental hormones used by the mycetophagous are ecdysone and 20-hydroxyecdysone.

The RIA performed did not show conclusively that the ecdysteroids used by the cecids are 20HE and its precursor ecdysone. High background level of non specific binding were seen in the assay. It was however clear that there was no immunoreactive material present in the fractions in which makisterone A would expect to have been found and although there was no evidence to suggest the presence of ecdysone some immunoreactivity was seen in the fractions which were eluted from the column at the same time as 20-hydroxyecdysone. In fact, the principal ecdysteroid immunoreactivity was seen in the early-eluting fractions (4 - 9 ml) that were more polar than 20-hydroxyecdysone, and in late-eluting fractions ($\geq$ 26 ml) that were more hydrophobic than ecdysone. These peaks could represent ecdysteroid metabolites or conjugates.

If this experiment was to be repeated further purification steps could be taken to ensure that the sample did not contain any contaminants; one of the problems however with extracting both sterols and ecdysteroids from paedogenetic cecids is that very minute samples are obtained from tissue which is very time consuming to collect.

Chapter 5

Larval Nutrition.

5.1 Introduction.

5.1.1 Viviparity.

The majority of insects are oviparous. They produce and lay eggs which contain a large amount of yolk which nourishes the embryo throughout development. This is necessary as the development of offspring in these insects takes place away from the mother. The nutrients in the egg are known as yolk or vitellus; they are usually made up of proteins, sugars and lipids.

Cecids do not produce eggs in the same way as oviparous insects; they are viviparous and the growth of their embryos takes place within the mother. Viviparity, as demonstrated by these insects, is a very unusual phenomenon. It is described as a form of parasitism between generations rather than between species by many authors (Wyatt 1961, Ivanova-Kasas 1965). During larval development the environment of the paedogenetic cecid larvae is that of the mother larva and their growth is at the expense of the maternal tissues. The result of this is that the nutrition of the mother larva and her offspring are very closely linked (Ivanova-Kasas 1965).

5.1.2 Larval nutrition in the Cecidomyiidae.

The first part of oogenesis in cecids is the only process during egg development which takes place within the maternal genital tract. As observed in this study (Section 2.3.2) after around 24 hours the development of the embryos switches to the haemocoel of the mother after the eggs are released from the ovary.

The eggs produced by a paedogenetic individual are described as being much smaller than those produced by the adults (Went and Camenzind 1980). This is not surprising, as those laid by an adult are fewer in number and will contain nutrient reserves to feed the embryos throughout the course of development. This is in contrast to the paedogenetic eggs which do not contain large amounts of yolk and which spend the majority of their development within the haemocoel of the mother.

In the first part of paedogenetic development the serosa and the yolk which is present within the eggs play an important role in embryonic nutrition. Each egg follicle is made up from an oocyte and a nurse chamber which are surrounded by a follicular epithelium.

Ivanova-Kasas (1965) described the main nutritive compound within the nurse chamber as being 'fat'; she states that the follicles themselves are permeable and are able to accumulate proteins from the surrounding maternal tissues. These proteins are first found in the nurse chamber but eventually find their way into the oocyte. In her work Ivanova-Kasas (1965) found that no proteinacious substances were stored within

the developing eggs and that all the proteins that passed across the follicular epithelium and were accumulated by the embryo were using in cell production.

Ivanova-Kasas (1965) discovered that the nurse chamber was rich in RNA and contains 'fat granules' but no carbohydrate. These lipids supply the oocyte with lipid precursors during the first stage of oogenesis. The size of the nurse chamber decreases proportionally as the follicle develops. By the end of oogenesis the nurse chamber has degenerated.

When the nurse chamber degenerates Ivanova-Kasas (1965) claims that the oocyte is nourished by the follicular epithelium. Using electron microscopy Junquera (1983a) confirmed that, at this time, the follicular epithelium is permeable to small molecules. Ivanova-Kasas (1965) suggests that nutrition of the embryo at this time occurs by nourishment passively penetrating the follicular epithelium and entering the developing embryo, not by any active assimilation of proteins within the developmental area. Ivanova-Kasas (1965) does not make clear the source of these nutrients, maternal tissue or maternal haemolymph.

As described in Chapter 1 the role of the follicular epithelium in these insects is not the usual role of chorion production. As described above it may have the role of synthesising some proteins and incorporating the proteins contained within the maternal haemolymph into the oocyte (Junquera 1983b). Although Ivanova-Kasas (1965) mentions the role of the follicular epithelium she raises doubt as to whether its role is one of actual protein synthesis or whether its structure simply allows the embryo

to assimilate nutrients produced by the maternal fat bodies contained within the haemolymph.

Following the degeneration of the nurse chamber a cuticle is produced by the embryo and a larval moult occurs within the follicular epithelium. By this time the nutrients available to the larvae are restricted to what remains in the mother larva's haemolymph and fat body reserves. Ivanova-Kasas (1965) describes these reserves as being made up of mainly glycogen and fat.

The larvae are unable to physically consume the mother larvae; firstly, because they are contained within the follicular epithelium and secondly because they have very specialised mouth parts designed for sucking the protoplast from delicate fungal hyphae and not for biting and chewing insect tissue. The question of how the larvae utilise the maternal tissue is raised.

Went (1982) describes the development of the offspring as being at the expense of maternal tissues. He describes how, under certain circumstances, embryos can degenerate and fail to develop. Went says that the numbers of embryos which fail to reach maturity is dependant on the nutritive state of the mother.

As the development of the embryo larva within the mother continues its dimensions increase and the pigmented spots of eyes become visible. During the course of paedogenetic development the increase in the size of the follicle to the fully grown offspring before emergence is around two hundred fold (Junquera 1983a). When the

offspring larvae emerge no part of the mother larvae is left remaining except the cuticle and the tracheal system (Ivanova-Kasas 1965).

The literature which will now be discussed concerns vitellogenesis, the process by which insects produce proteins which are inVolumeved offspring nutrition, and the tissues inVolumeved in this process, such as the fat body. The literature which exists regarding these topics almost exclusively concerns oviparous insects. There has been little research undertaken to establish the method of nutrition for viviparous, paedogenetic, larvae such as the Cecidomyiidae.

Embryonic development within the Cecidomyiidae is thought to be similar to that of internal parasites such as some of the Hymenoptera because the eggs are very small and contain limited mounts of reserved nutrients (Ivanova-Kasas 1965). It is fair to assume that processes similar to vitellogenesis take place in the paedogenetic Cecidomyiidae, but that they occur during parts of the life cycle different to those in the oviparous insects, in order to allow for the precocious production of offspring.

5.1.3 Vitellogenesis.

Vitellogenesis is the period of oocyte growth during which yolk accumulation occurs within insect eggs. The yolk or vitellus consists mainly of proteins, sugars and lipids. In oviparous insects vitellogenesis occurs in adult female insects before the eggs are laid since the nutrients needed for the developing embryos must be laid down in the egg before it leaves the mother (Valle 1993).

Vitellogenins are a superfamily of high molecular weight glycolipoproteins, translated as polypeptides of 215 - 260 kDa (Wyatt and Davey 1996). They are the precursors to the proteins found in the yolk of insect eggs. In most insects they are produced by the fat body (Raikhel and Lea 1983), and are released into the haemolymph (Girardie, Richard and Girardie 1996) from where the oocytes accumulate the yolk protein in the form of crystalline vitellin (Raikhel 1987, Izumi, Yano, Yamamoto, Takahashi 1994). In some insect species, including the higher Diptera, some vitellogenin production can also occur in the ovaries (Keeley 1994).

5.1.3.1 Triggers for vitellogenesis.

Endocrine and different physiological cues regulate the process of vitellogenesis in different insect species (Martin, Piulachs and Belles 1996). According to Valle (1993) vitellogenesis in insects is controlled by two hormones; ecdysone and juvenile hormone.

Many authors describe how, in many insect species including the locust, *Locusta migratoria* (Girardie, Richard and Girardie 1996) and the fly, *Calliphora stygia* (de Priester and van der Molen 1979), structural changes in the fat body cells have been observed during vitellogenesis. Many of these authors used the mosquito, *Aedes aegypti,* as the subject of their study as the timing of vitellogenesis can be manipulated easily in this insect by providing the female adults with the trigger of a blood meal (Deitsch, Dittmer, Kapitskaya, Chen, Cho and Raikhel 1995). Dortland and Esch (1979) used the Colorado potato beetle as vitellogenesis in this insect appears to be triggered by photoperiod.

In their review Wyatt and Davey (1996) describe how many authors attribute the start of vitellogenesis to a rise in the titre of juvenile hormone (JH) (e.g. Raikhel and Lea 1983, Craddock and Boake 1992, Valle 1993, Chinzei, Azumi, Miura, Matsuoka and Ando 1994). Wyatt and Davey (1996) suggest that it is a rise in the JH titre prior to vitellogenesis which promotes a response in the fat body to the rising physiological levels of 20-hydroxyecdysone. They claim that this initial rise in the levels of JH enables the fat body cells to undergo the ultrastructural changes mentioned above (and described at length in Section 5.1.6) including the proliferation of protein-synthesising apparatus. These changes prime the fat body cells and enable them to begin the protein synthesis required during vitellogenesis when the levels of 20-hydroxyecdysone start to rise following a decline in the levels of JH.

Many authors describe other cues for the initiation of vitellogenesis. It is possible that it is not these cues alone which trigger the process but that they cause a rise in the titre of JH. For example, in the kissing bug, *Rhodnius prolixus*, the trigger for the start of vitellogenesis is described as adult emergence and at this time both vitellogenin and storage proteins can be detected in both the fat body and the ovary (Chinzei, Azumi, Miura, Matsuoka and Ando 1994).

In *Drosophila silvestris,* Craddock and Boake (1992) found that a hormonal cue triggered vitellogenesis in adult females. This process could be significantly accelerated if the adult females were in the presence of males. They concluded that it is a behavioural trigger which leads to the induction of the hormonal cue.

5.1.3.2 Vitellogenins and vitellins.

Vitellogenins are phospholipoglycoproteins and are derived from precursors composed of a number of different subunits (Valle 1993). In her review, Valle (1993) gives the molecular weights of many insect vitellins and vitellogenins. The molecular weights of the native proteins range from 175 to 700 kDa and the weights of the denatured precursors and subunits range from 13 to 265 kDa.

The isolation of vitellogenins from insect fat body cells can sometimes be difficult; it can be confused by the fact that the fat bodies of many insects can secrete other proteins which are not related to vitellogenin. Different insect species can also have more than one vitellin. These can be differentially sequestered and can be found within different areas within the developing oocyte (Valle 1993).

Wyatt and Davey (1996) describe a number of proteins which are found in the fat body cells of different insect species which are similar to and are often confused with vitellogenins. These include: microvitellogenins of low molecular weight (around 31 kDa) which are found in the Lepidoptera, silk worm 30 kDa proteins found in *Bombyx mori*, and the locust protein jhp21 (21 kDa).

Many of the proteins found in the insect haemolymph are not vitellogenic but are hexameric storage proteins that accumulate in the final larval instar to provide amino acids for the construction of adult structures. Wyatt and Davey (1996) state that the

majority of the proteins found in the eggs of insects is vitellin; this is the yolk protein which is produced from vitellogenin.

5.1.4 Fat body.

The arrangement of the fat body within the insect varies from species to species (Haunerland and Shirk 1995). The cells are highly tracheated and can lie in ribbons or in single cells, these can be distributed from the head to the abdomen (Smith 1968, Dean, Locke and Collins 1985). The cells of the fat body are suspended in the haemolymph of the insect and as such they are in contact with the proteins, nutrients and hormones that are present there. Keeley (1985) describes the fat body as being structurally organised in order to provide maximum exposure to the haemolymph.

Although its name suggests that it is a storage tissue only for lipids, the fat body is actually a very complex and versatile organ storing lipids, protein and carbohydrate (Price 1973). It is inVolumeved in the synthesis of haemolymph proteins (Wyatt 1980), Smith (1968) and Locke (1984) also describe it as also being a site for intermediary metabolism and compares its function to that of the vertebrate liver. Raikhel and Lea (1983) include the regulation of blood protein and sugars in their list of functions of the insect fat body.

Insect fat body cells typically have rounded nuclei, fat deposits, mitochondria, a Golgi complex, endoplasmic reticulum, glycogen granules and many ribosomes (Price 1973). Food reserves are seen to accumulate in the fat body during the larval phase in most

insects, and over time the number of inclusions in the cells increases incorporating more glycogen, fat and protein.

The carbohydrate stored in the fat body cells is usually in the form of glycogen granules while the major storage lipid is thought to be a triglyceride. These food reserves enable the insect to survive for long periods of time without food during periods such as pupation and diapause. The amount of nutrient reserves within the fat body cells will vary depending upon the nutritional state of the individual insect (Smith 1968). Price (1973) describes experiments performed to gauge the effect of starvation on the fat body; typically it caused the disappearance of glycogen, RNA and mitochondria in larvae of the blood-sucking bug *Rhodnius prolixus* and the blowfly larva *Calliphora stygia.*

Smith (1968) describes studies on the lepidopteran *Calpodes ethlius*, in which fine structural changes are seen in the fat body. In this insect lipid and glycogen stores are seen to increase during the larval instars as is the presence of rough endoplasmic reticulum (RER), which provides the larvae with the ability to synthesise proteins. The storage of these nutrients increases as pupation approaches in order to provide the insect with reserves for this time of tissue reorganisation. Along with the changes in the ultrastructure certain other changes are seen in the fat body cells of *Calpodes ethlius* larvae approaching metamorphosis. These include the appearance of urate crystals, lysosomes and protein granules (Dean *et al.* 1985).

Changes are seen in the structure of the fat body in each larval stage in many insects (Locke 1980). de Preister and van der Molen (1979) studied the ultrastructural changes which occur in the fat body of premetamorphic *Calliphora stygia* larvae. Initially these fat body cells are rich in ribosomes and RER which synthesise and export haemolymph proteins. Following a decline in the production in the haemolymph protein, calliphorin, the amount of protein-producing apparatus seen in the fat body decreases. At this point Golgi bodies can be seen to be budding off presumably to produce protein vesicles for the period of pupation. After this period of organelle degradation, immediately prior to pupation, new evidence of protein production is seen. It is thought that this is needed to produce the enzymes needed for tissue reorganisation during pupation.

The work done by Haunerland, Nair and Bowers (1990) points to there being structural differentiation between different areas of the fat body in the bollworm, *Heliothis zea*, with the perivisceral fat body being responsible for storage while the peripheral fat body cells are the only ones capable of biosynthesis. They state that in Diptera these functional histotypes are arranged along the anterior-posterior axis and that they differ in the larval and adult stages. Regional differentiation can take many forms with certain areas having different colourations, gene expression patterns, quantities of storage proteins and glycogen content.

In their review article Haunerland and Shirk (1995) describe how the regions within the fat body which have differing functions also have unique morphologies. However, in conflict with this theory, the authors also present some evidence to suggest that the

insect fat body is a single pleiomorphic tissue which simply shifts functional activity throughout development. They conclude that there is evidence to suggest that there is both change with developmental stage and regional differentiation in structure and function.

The idea that there may be areas of the fat body which may be functionally different may explain the way in which the fat body appears to produce proteins which are released into the haemolymph while at the same time sequestering proteins from the haemolymph (Dean, Locke and Collins 1985).

Raikhel and Lea (1983) describe how the function of the fat body is hormonally controlled throughout the developmental stages of an insect's life cycle. Price (1973) describes how the steroid hormone ecdysone and juvenile hormone have been found to induce protein production in the fat body of *Calliphora stygia.*

Price (1973) documents the many descriptions of the storage of protein in the fat body; he describes these as proteinaceous spheres. He describes the appearance of these spheres being reliant on the stage in the life cycle and being controlled hormonally. He concludes that rather than these proteins being solely produced within the fat body cells the majority of them are sequestered from the haemolymph. The converse is also true; there are a number of proteins which are produced in the fat body and are released into the haemolymph. He states that many workers have observed changes in the protein content of the insect haemolymph through the course of development.

Price (1973) describes how many of the proteins which occur in the fat bodies of insects such as the cockroach, *Periplaneta americana*, are conjugated with lipids or carbohydrates. In the case of the cockroach it is the lipoproteins which are transported into the haemolymph.

Haunerland *et al.* (1990) describe the storage proteins produced in the larval stage in the bollworm, *Heliothis zea*, as being those necessary to produce the amino acids needed for the production of adult structures and describe the proteins being produced by the fat body in the adult as being those necessary for reproduction. Caglayan and Gilbert (1987) describe the storage proteins produced by fifth instar *Manduca sexta* larvae as being the amino acid reserves for adult metamorphosis. Dutkowski (1974) describes a decrease in the stored nutrients contained within the fat body cells of the wax moth, *Galleria mellonella*, at the start of the pharate-pupa stage which continues into the adult insects.

Smith (1968) and Price (1973) describe the presence of lysosomes in the fat body cells of many insect species. They state that these vary in structure but that they all contain hydrolytic enzymes. These enzymes are very important for the cell remodelling which occurs during metamorphosis in order to produce adult tissues. Whether the tissues within the fat body are completely remodelled, or whether certain areas retain their functional specialisation is unknown. Little work has been done on the structure of pupal tissues or the fate of larval fat body cells during pupation because of the difficulties experienced in handling the tissue at this stage (Haunerland and Shirk 1995).

In certain insect species such as Collembola and Lepidoptera the fat body acts as a storage organ for waste metabolites such as uric acid, the major end-product of nitrogen metabolism. These waste products are stored in the form of crystals in fibrous protein granules within the fat body cells. It is thought that these crystals may provide a source of nitrogen for the synthesis of new tissues during metamorphosis (Smith 1968). Buckner, Caldwell and Knoper (1985) discuss the storage of uric acid in the fat body cells during pupation in the tobacco hornworm, *Manduca sexta*.

Fat body is described as being present in adult oviparous insects by Smith (1968). He states that at this stage the fat body is very important as it still has the ability to produce proteins and fulfils the function of nutrient supply for egg production. During this stage the fat body provides a very important nutrient source for egg production, especially in insects which do not feed as adults. For this reason in many insect species the fat body is larger in females than in males (Smith 1968). Johnson and Butterworth (1985) describe the fat body cells of female *Drosophila melanogaster* as having a higher proportion of stored lipid in comparison to that of males; they link this with the reproductive function of the fat body in the female insects.

Fat body tissue is renowned for being difficult to work with. The loose structure of the fat body cells makes it especially difficult to prepare for histological research. It is a difficult tissue to fix and its high lipid and vacuolar content makes it difficult to section (Dutkowski 1974, Locke 1984 and Dean, Locke and Collins 1985). Its changing morphology makes observations of cell structure very complex and in certain species,

such as *Heteropeza pygmaea*, this is accompanied by a gross change in structure when the cells of the fat body tissue dissociate into single cells prior to pupation (Richard, Arnim and Gilbert 1993, Haunerland and Shirk 1995). The isolation of fat body tissue from haemolymph and other tissues during these times is very difficult.

5.1.5 Fat bodies in the Cecidomyiidae.

The paedogenetic larvae of the Cecidomyiidae have pronounced fat bodies. Ribbons of fat can be seen clearly in both *H. pygmaea* and *M. speyeri*; the literature is not clear about the role the fat body plays in the development of these larvae but does refer to its nutritive function during *in vitro* culture of eggs (Went 1971). The questions arise, are adult proteins produced by the fat bodies of paedogenetic Cecidomyiidae larvae which produce their young while still in the larval stages? Do those insects destined to reproduce paedogenetically, and therefore never to produce adults, still produce the proteins needed during metamorphosis and as adults?

In his paper Went (1971) establishes the importance of the nutritive function of the fat body cells by culturing *H. pygmaea* embryos *in vitro* using the fat body cells as a nutrition source. Went exposed paedogenetic mother larvae to X-rays before they gave birth to their offspring. These offspring developed normally but did not produce offspring of their own; they were used as donors of fat body cells and haemolymph. *H. pygmaea* was the ideal cecid species for this study because before the end of their development their fat body lobes dissociate into individual cells. Eggs from a normal mother larvae were added to this haemolymph containing the fat body cells and their development was monitored. The eggs produced normal embryos and then larvae in a

very similar time to individuals developed *in vivo*. He concludes that the fat body must have a nutritive function as during the course of *in vitro* development of the embryos the fat body cells decrease in size. They also changed from being opaque to transparent. He investigated whether these nutrients could have been provided by the haemolymph by culturing embryos in haemolymph alone without fat body cells but these embryos did not develop to maturity.

Went (1971) also observed that eggs cultured *in vitro* did not thrive if the fat body cells were damaged in any way. He concluded that some agents from the fat body must damage the eggs and embryos in some way. This suggests that some kind of lysosomal function is performed by the fat body. Went describes the culture of cells in medium derived from individuals of the same species as being a disadvantage due to its undefined nature. Although this is certainly the case this work did enable him to isolate the importance of fat body cells in paedogenetic development of Cecidomyiidae. How are nutrients transferred from the fat body to the paedogenetic larvae? Ivanova-Kasas (1965) suggested that during the development of the paedogenetic larvae the absorption of nutrients from the mother's fat body may be due to the action of proteolytic enzymes. Alternatively, the active export of proteins from the fat bodies may be involved.

Throughout the developmental process in the paedogenetic Cecidomyiidae the content of lipids in the egg and then in the embryo increases even when the increase in size is taken into account. Within the offspring larvae the fat moves from reserves in the syncytium to the developing fat bodies (Ivanova-Kasas 1965).

As the egg, and then the embryo develop changes are seen in the maternal larva's nutrition. She will reduce her food intake and become motionless. While this is happening changes can be seen in the structures within her body. In *M. speyeri* the fat bodies of the maternal insect can be seen to lose their orange colour and in *H. pygmaea* the fat body disintegrates into isolated cells (Ivanova-Kasas 1965, Went 1971). These cells disperse into the haemocoel presumably in order to nourish the developing offspring. This process can be compared to yolk production in oviparous insects.

Junquera (1983a) outlines the important role played by the follicular epithelium in the Cecidomyiidae in incorporating haemolymph proteins into the oocyte. The ability of this structure to produce protein itself is investigated as in other species it is able to produce proteins as its role is to produce the chorion. He concludes that there is no evidence to suggest that the follicular epithelium is able to produce its own protein and that it simply facilitates the uptake of proteins from the haemolymph.

The absence of a chorion in the paedogenetic cecids is an important adaptation in terms of larval nutrition, as it enables the developing embryos to remove nutrients from their environment and makes them less reliant on the nutrients laid down within the egg. If the lack of a chorion allows the process of oogenesis to be completed more quickly, the lack of a chorion may be an explanation for the ability of species such as *H. pygmaea* to speed up oogenesis and to produce offspring precociously.

5.1.6 Role of fat body in vitellogenesis

In oogenic female adults in oviparous species the most important role of the fat body is the synthesis of vitellogenin (Raikhel and Lea 1983). The proteolytic processing of the vitellogenin precursors also occurs within the fat body (Valle 1993).

The production of vitellogenin inVolumeves extensive remodelling within the fat body (Dortland and Hogen Esch 1979, Valle 1993). During vitellogenesis, in order to produce vitellogenin, the fat bodies in insects undergo a number of changes; they change from structures which store lipids and glycogen and become organs for protein synthesis.

In ultrastructural studies carried out on the adult mosquito, *A. aegypti*, Raikhel and Lea (1983) have described the changes in the fat body occurring in three main stages. First is a previtellogenic phase during which the cells in the fat body prepare to synthesise vitellogenin and have a large number of lipid inclusions, the enlargement and activation of the nuclei, proliferation of ribosomes and rough endoplasmic reticulum (RER), the production of Golgi bodies and the occurrence of invaginations of the plasma membrane. In phase two, the vitellogenic phase during which the fat bodies contain a large amount of rough endoplasmic reticulum, the fat bodies produce the largest amount of protein and secretion granules appear in the Golgi apparatus. Phase three is the time when the fat bodies begin to deteriorate and are full of lysosomes (Valle 1993).

In the final phase of fat body degradation the vitellogenin secretory granules within the fat body fuse with lysosomes containing the lysosomal enzyme cathepsin D. Many of these stages have also been described for other insects species such as *Locusta migratoria* and *Leptinotarsa decemlineata* (Dortland and Hogen Esch 1979).

Many authors including Deitsch *et al* (1995) describe the final stage of vitellogenesis in many insect species. During this phase the cell machinery used for protein production undergoes a programmed degradation after the proliferation of lysosomes containing enzymes.

In adult oviparous insects the proliferation of protein synthesising apparatus described above is particularly important in the previtellogenic stage as much of this apparatus will have been lost in the fat body during metamorphosis when the fat body acts solely as a storage organ (Wyatt and Davey 1996).

In *Leptinotarsa decemlineata* reared under long-day conditions the fat body becomes highly specialised for protein synthesis. It becomes rich in RER and ribosomes. In the study by Dortland and Hogen Esch (1979) this change in the ultrastructure of the fat body coincided with the production of large amounts of vitellogenin, the yolk precursor.

5.2 Materials and Methods.

5.2.1 Ultrastructural study of the fat bodies of *M. speyeri.*

Paedogenetic *M. speyeri* larvae were kept in optimal conditions on fungal plates of three day old *Chondrostereum hirsutum* (as described in Section 2.2.1). Four different ages of insect were used in the experiment: 1 day old, 3 days old, 5 days old and pupae.

It proved difficult to achieve adequate fixation and embedding of the larval tissues. Some of the procedures tried are described in the Results section. The fixation method finally found to give the best results was as follows:

The larvae were taken from the plate and fixed using a method adapted from Meats and Tucker (1976) and Mahowald and Stoiber (1974), in a solution of 2% paraformaldehyde (Agar Scientific Ltd.) and 2% glutaraldehyde (Agar Scientific Ltd.), in a 0.1M solution of phosphate buffer, at pH 7.45, for 2 hours. Each insect was dissected under the fixative to ensure that the fat bodies were fixed thoroughly.

The samples were rinsed in a washing buffer containing 0.1M phosphate buffer with 4% sucrose and then postfixed for one hour using 2% aqueous osmium tetroxide. The insects were then washed in distilled water and dehydrated using an acetone series (BDH). They were left in 100% acetone (dried using molecular sieve) for one hour to ensure the removal of any remaining water.

The larvae were then embedded in Spurr's resin (Agar Scientific Ltd.) (Spurr 1969) and polymerised at 70°C for 8 hours. After polymerisation the resin blocks were sectioned at 60 nm on a Reichert OMU3 microtome using glass knives (TAAB) and collected onto copper \ palladium grids (Agar Scientific Ltd). Details of the solutions used during preparation are given in Appendix 2.

The sections were stained using uranyl acetate and Reynolds' lead citrate (Reynolds 1963). They were viewed using a Jeol JEM 1200 EX electron microscope (Jeol, Tokyo, Japan).

5.2.2 Insect tissue preparation and protein content determination.

In order to prepare tissue for protein analysis, individual *M. speyeri* larvae were placed under a dissecting microscope (Olympus), on a depression slide and covered with 2µl of Tris (Sigma), 0.02M, buffered saline (NaCl), 0.5M containing the protease inhibitors; EDTA (1 mM) and PMSF (1 mM), pH 7.4. The insects used were at the following stages: 1 day old, 3 days old, 5 days old and pupae.

The cuticle was torn carefully from the head and anus ends of the larvae using two pairs of watchmaker's forceps. The gut and the mouthparts were removed with the head and discarded along with the empty cuticle. At this stage the buffer remaining on the slide contained only the haemolymph, the fat bodies and, in the case of the 3 and 5 day old larvae, the embryos.

The pupae used were destined to produce adults and at this stage did not contain any eggs and the 1 day old larvae contained paedogenetic eggs at the follicular stage which were too small to collect.

A fine glass needle was used to draw off the buffer on the slide which contained the haemolymph; care was taken not damage or remove the embryos and fat bodies from the slide. This fraction was collected in a 0.5 ml microcentrifuge tube and stored at -40 °C. Fresh buffer was added to the slide which still contained the fat bodies and the embryos; these were then carefully removed and placed into separate microcentrifuge tubes containing fresh buffer. This process was repeated until the tissue from around 10 insects from each stage had been collected. The tubes containing the fat body and the embryos for each stage were macerated, centrifuged to remove any debris and the supernatant was collected.

The protein content for each sample was estimated using a Lowry assay (Lowry, Rosebrough, Farr and Randall 1951). The absorbence of the sample solutions was measured on a plate reader (Dynatech) at 620 nm. The absorbances were compared to those for a standard series made from a Bovine Serum Albumin solution (Sigma) diluted in 0.25M sucrose. See Appendix 3 for details of the assay solutions.

5.2. 3 SDS-PAGE gel of insect tissue samples.

The proteins in the 10 samples: day old haemolymph, fat body and embryo, 3 day old haemolymph, fat body and embryo, 1 day old haemolymph, fat body and pupal fat

body and haemolymph, were separated on a 10% acrylamide resolving gel using a BioRad Mini - PROTEAN II system.

Each protein sample was dissolved in Laemmli buffer (Laemmli 1970). These were then boiled in a hot water bath at 100 °C for three minutes before being centrifuged to remove any precipitation, 2.4 μg of each sample was then loaded into the lanes of the gel.

The samples along with a marker protein sample were then electrophoresed, at room temperature, at 200 V for approximately one hour until they reached the end of the resolving gel. See Appendix 4 for details of the solutions used.

Following electrophoresis the polypeptide bands on the gels were stained using a BioRad Silver Stain Plus kit. This technique allows the visualisation of protein concentrations as small as one nanogram.

The gels were covered with clear film and dried overnight at room temperature, using a BioRad vacuum drier. Densitometric analysis was then performed on the protein bands on the stained gels using a GS-670 BioRad laser densitometer linked to the image analysis programme Microsoft Molecular Analyst.

5.2.4 Observations of fat bodies *in vitro.*

Fat body tissue was removed from three day old larvae using the technique described in Section 5.2.2.. It was washed thoroughly using Tris-buffered saline (0.02M Tris,

chosen for this experiment as it is at this age that the embryos within the larvae make the most significant increases in size.

Fat body tissue from 3 larvae was placed in each of 8 microcentrifuge tubes all containing 15 µl of Schneider's sterile insect tissue cell culture medium (Sigma). After 5 minutes, 30 minutes, 1 hour and 2 hours samples of the culture medium were removed and subjected to SDS-Page electrophoresis (as in Section 5.2.3).

Initial experiments had been carried out using TC 100 (Sigma) culture medium to bathe the cells. Although protein bands were seen on the gels produced from the fat body cells in this medium, it was decided that a culture medium designed specifically for dipteran cells would be more suitable and Schneider's medium was chosen.

After staining the gels with BioRad Silver Stain Plus kit the sample lanes were examined for the presence of proteins which had been exported by the fat bodies during the experiment. Solutions of Schneider's medium which did not contain insect tissue were used as controls and the samples taken from the 3 day old, fat body homogenate and haemolymph used in Section 5.2.2 were used for comparison.

5.3 Results.

5.3.1 Fixation.

Many methods were used to fix the fat body tissue of *M. speyeri*. Initially whole insects were used; it was thought that this would enable us to study the fat body *in situ*. This did not allow proper fixation of the fat body tissue; large areas of the tissue were lost on sectioning as the resin had not penetrated adequately.

A number of fixative systems were used on dissected fat body tissue in order to try to fix the lipids within the fat body. These included adding 0.05% malachite green to the initial 2% glutaraldehyde step and using 1% osmium tetroxide as well as 2% glutaraldehyde in the first fixative step. The system which gave the best results on the excised lipid tissue is that described in section 5.2.1 using an initial fixative solution containing 2% glutaraldehyde and 2% paraformaldehyde.

5.3.2 Ultrastructural study of the fat bodies of *M. speyeri*.

Attention was paid to the fat body cells and the way in which they change during the 6 days of the paedogenetic life cycle. Photographs were taken of large areas of the cells; these prints were assembled as montages in order to create detailed pictures of entire cells at a high magnification.

Photographs were taken of the cytoplasm within the fat body cells; particular attention was paid to the structures present within the cytoplasm with a view to determining the processes occurring with the cells at different stages within the life cycle. Attention was paid to the boundaries of the fat bodies in each case in order to establish whether

the fat body cells were actively exporting products or incorporating substances from the surrounding haemolymph.

The larvae studied were paedogenetic larvae of 1, 3 and 5 days old and pupae of unknown age. The cuticles of each insect had been torn open prior to fixation in order to ensure that the fat body tissues were fixed adequately; in all the cases except one this had resulted in the loss of the haemolymph and, in the case of the paedogenetic larvae, the developing embryos. In the case of one 5 day old larva it was discovered that 2 embryo larvae had remained within the cuticle. These embryos were found to be lying directly adjacent to the lobes of fat body within their mother-larva.

The embryo tissue that had remained within the maternal cuticle, in the case of these 5 day old larvae, had been sufficiently well fixed to allow useful pictures to be taken; this was unusual as problems had previously been experienced in fixing the tissues of embryo larvae which still retained their own cuticle. This success enabled the tissue of the embryos to be studied and any interactions between the tissues of the mother larva and the embryos to be observed.

At least two insects were examined from each stage and sections were viewed from different areas of each insect. The fat body tissue from each section examined appeared to be the same for each stage. There was no evidence of regional variation of fat body function in the insects studied, although within each cell the cytoplasm often had distinct areas containing different organelles. The tissues shown in Figures 5.1 - 5.20 are typical of each of the different stages examined.

5.3.2.1 Observations of cytoplasm structure at the different stages.

Fat body from embryo larva.

The two embryos examined were within a day 5 paedogenetic larva and were therefore only one day short of hatching. Fixation of this tissue was not of the highest standard, presumably because of poor penetration by the fixative of the embryonic cuticle. However useful information can be derived .

Figures 5.1- 5.4 and Montage 1 show fat body cells seen in an embryo larva. The cells contained many lipid droplets (Figure 5.1) which did not appear to be membrane bound (Figure 5.2). The cytoplasm appeared to have 2 main regions; these can be seen in Figure 5.2. Type I cytoplasm contained densely packed glycogen granules and few other organelles, while Type II cytoplasm contained many of ribosomes, mitochondria, RER and less densely packed glycogen granules (Figures 5.3 and 5.4). The nuclei seen in Figure 5.1 contained dark-staining nucleoli.

Fat body from day 1 paedogenetic larva.

Figures 5.5 - 5.8 and Montage 2 show the fat body cells of a 1 day old paedogenetic larva. The cytoplasm within these cells was less densely packed than that seen in the embryonic fat body cells. The fat body cells of the 1 day old larvae also contained large lipid droplets. The better fixation of this tissue and later samples reveals the opening of cellular membranes into the boundary of the lipid droplets (Figures 5.5 and 5.9). Unlike those in the later stages lipid droplets of one day old fat body did not contain any darkly staining spherical bodies. The cytoplasm contained many mitochondria

(Figure 5.7), ribosomes, many areas of densely packed RER and areas of glycogen granules (Figures 5.5 - 5.7).

Fat body from day 3 paedogenetic larva.

Fat body cells from 3 day old paedogenetic larvae, shown in Figures 5.9 - 5.12 and in Montage 3, were similar to those of the one day old larvae. The main difference was that the cells contained fewer areas of cytoplasm rich in ribosomes and more large areas that are rich in glycogen granules. Montage 3, and Figures 5.12 and 5.10 show that although there were many lipid droplets, some of them were empty and had been filled with glycogen containing cytoplasm. Some of the lipid droplets appeared to contain small, spherical, electron-dense bodies (Montage 3). The fat body cells of the 3 day old larvae did contain areas of cytoplasm containing ribosomes and mitochondria but these tended to be concentrated at the cell margins (Figure 5.12) or adjacent to the nuclei or lipid droplets (Figure 5.9). The intercellular spaces in the three first stages (embryo, 1 and 3 day old larvae) appeared to be very narrow.

Fat body from day 5 paedogenetic larva

The fat body cells of the 5 day old paedogenetic larvae are illustrated by Figures 5.13 - 5.16 and in Montage 4. These cells still contained a number of lipid droplets although many were empty and had been filled with glycogen containing cytoplasm (Figure 5.15). Dark-staining electron-dense, spherical bodies could be seen in most of the remaining lipid droplets (Montage 4). These spherical bodies appeared to accumulate around the edge of the lipid droplets (Figures 5.16 and 5.15).

Although very much reduced there were areas of ribosomal cytoplasm and small quantities of RER. The cell boundaries in these cells were well defined; this was due to the fact that there were wide spaces which created channels between the cells (Figure 5.15). Adjacent to the cell boundaries dense areas of non-glycogen cytoplasm could be seen; these areas contained many mitochondria. Intact nuclei could be seen in the cells of the 5 day old larvae. As in previous stages these nuclei contained densely staining condensed chromatin, but did not appear pyknotic (Montage 4).

Fat body from pupa.

Figures 5.17 and 5.20 and Montage 5 show the fat body cells seen in the pupal samples. This tissue strongly resembled that of the 3 day old larvae in many ways. It contained many lipid droplets, few of which contained electron-dense bodies. The cytoplasm had areas containing glycogen granules and areas which contained mitochondria and some ribosomes, although the numbers of ribosomes were very much reduced in comparison with the embryonic and larval stages.

The nucleus in the pupal stage (Figure 5.18, Montage 5) appeared to be different to those seen in the embryonic and larval stages. It appeared to be generally darker with less obviously differentiated chromatin. The main difference in the pupal tissue to that of the other stages was the presence of complex, multivesicular granules, shown in Figure 5.20. These structures were not seen at any other stage. It is thought that these bodies were lysosomal in origin their role may be to break down unwanted areas of the cell during pupation or perhaps to digest nutrients which are imported from the haemolymph.

Embryo - larva interface.

As previously mentioned it was possible to study in the interface between an embryo larva and the maternal fat body from a 5 day old, paedogenetic larva (Montage 6). It has been found on studying sections of whole paedogenetic larvae that the embryos developing within the mother larva's haemolymph are often closely associated with the maternal fat body (Section 2.3.3). One possible interpretation of this would be that nutrients produced within the fat body are transferred directly to the embryo.

On examination of the interface between the maternal tissue and the embryo no evidence of any direct transfer of nutrients could be seen. The maternal fat body cell cytoplasm adjacent to the embryo appeared to have receded somewhat (Montage 6) and the embryonic tissue was completely surrounded by the embryonic cuticle and the follicular epithelium. As there is no evidence of nutrients being passed from the maternal fat body to the embryo directly, it must be assumed that any lipids or vitellogenic proteins secreted by the maternal fat body, must be absorbed by the embryo indirectly from the haemolymph.

5.3.2.2 General observations.

The fat body cells of the insects examined appear to be relatively uniform in structure in any one stage of development. The fat body seems to be pleiomorphic in nature with differences in structure being seen between the tissues of insects of different ages rather than differences existing between the tissues from different regions within one individual.

The fat body in *M. speyeri* appears to fulfil a role of nutrient storage. There is much evidence of lipid storage in all the stages examined; this is in the form of lipid droplets which are contained within the cytoplasm. There is evidence to suggest that there is a decline in the amount of lipid stored within the fat body cells as the age of the larvae increases. Many of the lipid droplets of the 3 and 5 day old larvae had apparently disappeared and been replaced by glycogen-containing cytoplasm. There was some evidence of smooth endoplasmic reticulum (SER) in certain fat body cells, but this did not seem to be sufficient to account for the large quantities of lipid which appeared to be present from the embryonic stage.

The other major stored nutrient appears to be glycogen. This could be seen stored in the fat body cells of all ages, including the 5 day old insects, in the form of typical glycogen granules. In the cells of all stages there seemed to be areas of specialised cytoplasm, densely packed with glycogen granules. The glycogen granules in the embryonic tissue appeared to be the most densely packed. This may not be due to the fact that there are more glycogen granules present at this stage simply that the cells are not as large causing the glycogen to appear more densely packed.

There was no evidence of protein storage in the form of electron-dense, membrane bound vesicles in any of the larval stages. There was however extensive evidence of protein synthesis. In many of the sections (e.g. Figure 5.1) prominent nucleoli could be seen in the nuclei and in all the stages, although less in the pupal tissue, there were

many ribosomes clearly visible. Some of these could be seen as free ribosomes while many were associated with RER.

No evidence could be seen of protein export from the fat body cells. Although the cell boundaries were studied at length (Montages 1 - 5), no evidence was found of exocytosis and within the cytoplasm there were no obvious Golgi bodies and no evidence of the proteins which were being produced being packaged into vesicles. There was no evidence of any lysosomal activity in any of the larval stages to suggest that the fat body cells were broken down in order to release proteins into the haemolymph. In the case of the 3 and 5 day old fat bodies (Montage 3 and 4) there were some signs of disruption to the cells around the boundary. It is possible that this may represent protein export but this phenomenon was by no means common in the sections examined and could have been caused by mechanical damage during tissue preparation.

In many cases the lipid droplets contained spherical electron-dense bodies within them; these dark bodies usually appeared at the edge of the lipid droplets and were relatively uncommon in the embryonic, 1 and 3 day old tissue but were seen more regularly (more than 1 per lipid droplet) in the large larvae. The nature and role of these bodies was not clear.

The nuclei seen in all the embryonic and larval stages were similar. In some cases a large nucleolus was visible (Figure 5.1) but this was not always the case depending upon the plane of the sections. In some sections it was possible to see more than one

area of nuclear material in a single cell, probably indicating that the nuclei are not always spherical and can have complex shapes with lobes protruding into the surrounding cytoplasm. In many of the pictures it is possible to see chromatin dispersed within the nuclei. At no stage in the *M. speyeri* larvae examined did the nuclei appear pycnotic. Even in the fat body cells in the 5 day old larvae, which were due to die the next day, there was no sign of impending cell death.

As described by Smith (1968) the fat body of insects is a highly tracheated tissue. A high degree of tracheation is necessary to provide an oxygen supply to this metabolically active tissue. On dissection it became clear that the lobes of the fat body in *M. speyeri* were closely associated with large tracheae, and on examination of the sections of the fat body tissue it became clear that some tracheae actually pass through the fat body cells. These tracheae can be clearly seen in many of the photographs (e.g. Figure 5.13).

There was no evidence of any waste products of metabolism being stored in the fat body. The fat body cells of some other insects act as a storage site for urate crystals, a by-product of nitrogen metabolism (Smith 1968). No membrane-bound vesicles containing waste products or urate crystals could be seen within the fat body cells of the *M. speyeri* larvae examined.

The tissues of the fat body are notoriously difficult to fix (Dean, Locke and Collins 1985). This is due to the fact that they have high lipid content, often contain phagic vacuoles containing enzymes which can destroy cell structure if they are damaged

during tissue preparation, and because they contain different kinds of vacuoles all of which require different types of fixation. The method described in 5.2.2 gave good fixation of the fat body cells and the organelles contained within the cytoplasm. One area of difficulty experienced in the preservation of this tissue was the fixation of the many lipid droplets contained within the cytoplasm, and three artefacts of preparation can be seen in these organelles.

One artefact of the fixation process is visible in Figures 5.9 - 5.11; fixation and polymerisation of the lipid droplets has apparently caused the vesicles to shrink away from the surrounding cytoplasm. This has created a light-coloured ring to appear around the lipid droplets. Knife marks that have been made during sectioning of the tissue can be seen in the lipid droplets of a number of samples; this is due to the fixed and polymerised lipids being harder than the surrounding tissue.

In Figures 5.1- 5.4 and 5.13 - 5.16 (the embryo and 5 day old fat bodies) the solvents used in the tissue preparation have dissolved the lipids contained within the cytoplasm leaving what appears to be empty vesicles. It may be the case that this only occurred in the 5 day old larvae and the embryonic tissue because these were the only stages in which the lipid vesicles contained lipid alone. Perhaps in the other stages the lipid within these vesicles was conjugated with protein so that after processing these stages, although the lipid may have been removed, the protein still remained. This might explain why after staining in the pupal, 1, and 3 day old larvae the lipid vesicles appear grey in colour, while in the embryonic and 5 day old tissue the vesicles are empty and appear as such.

5.3.3 Insect tissue preparation and protein content determination.

The method that was developed and is described in Section 5.2.2 enabled different parts of these small insects to be separated, with minimum contamination between samples. The protein assay described in 5.2.2 enabled the protein content of the samples to be quantified.

The concentration of protein for individual insects could not be determined as it was impossible to gauge the efficiency of the extraction technique. The figures obtained using the protein assay technique described above could, however, be used to estimate the quantity of protein in the remaining samples taken from the tissue of the dissected larvae. This made it possible to load similar quantities of protein into each lane of the SDS-PAGE electrophoresis gel.

5.3.4 Minigels of all parts of the insect.

SDS-PAGE gels were used, as described in 5.2.3 to separate the proteins obtained from the *M. speyeri* tissue samples prepared using the method described in 5.2.2. Figure 5.21 shows a photograph of the gel produced.

Figure 5.21 shows clearly that many different proteins are contained within the samples. It is clear from the protein gel that the different parts of tissue from the *M. speyeri* larvae contained many similar proteins but that also different proteins were expressed at different stages in development.

Densitometric analysis enabled the allocation of an estimated molecular weight to all of the bands on each gel. Figure 5.22 shows the molecular weight of each band on the gel pictured in 5.21. The five most five most strongly expressed proteins from each tissue are highlighted on the graph.

In each of the samples there was a strong double band seen at around 66 kDa. This band is particularly noticeable on the sample collected from the 3 and 6 day old larval haemolymph and in the pupal samples. There is also a high molecular weight protein which can be seen in these samples on the gel at around 140 kDa.

A number of low molecular weight proteins of below 30 kDa can be seen in all the samples. These bands appear to be particularly clear in the pupal samples and in the samples taken from the 1, 3 and 6 day old haemolymph and the 6 day old embryo samples.

It has been a working assumption of this study that vitellin and vitellogenin, or whatever protein is produced by the mother larva to nourish the embryos, in the Cecidomyiidae is produced by the fat body and released into the haemolymph from where it is taken up by the embryos. As has been mentioned already the paedogenetic eggs produced by the mother larvae are very small (Went and Camenzind 1980) and only begin to increase significantly in size during embryogenesis which occurs when the mother larvae are between one and three days old.

It would seem reasonable that proteins synthesised for larval nutrition would be manufactured by the fat bodies of the 1 and 3 day old mother larvae, enter the haemolymph and be accumulated by the embryos. There are many proteins within the range 200 - 58 kDa which can be seen on the gel in Figure 5.21; these would match the criteria given above for proteins produced for embryonic nutrition. This range includes the values given by Valle (1993) for the denatured molecular weights of vitellin and vitellogenin subunits and precursors in the lower Diptera (58 - 211 kDa). The majority of the bands which may represent these proteins lie within the range 66 - 29 kDa on the gel.

5.3.5 Observations of fat bodies *in vitro*.

Fat body cells from three day old *M. speyeri* larvae were incubated in Schneider's tissue culture medium for varying periods of time. SDS-PAGE gels were used to separate the proteins produced by the fat body tissue while in the culture medium. Figure 5.23 shows two gels produced using the method described in Section 5.2.4.

Variation can be seen between the two gels illustrated in Figure 5.23. This can be attributed to a variation in the amount of protein loaded onto each lane of the gel on each occasion. Although each lane contained the total protein obtained from the fat body tissue from three larvae, the staining technique used is very sensitive and will highlight any variation present between the samples.

On examination of the protein band patterns produced in the lanes seen in Figure 5.23 it is clear that they match the bands produced by the sample of haemolymph from three

day old larvae rather than the fat body cell homogenate (Figure 5.21). It is therefore not the case that the proteins obtained from the culture medium in this experiment may be accounted for by proteins from fat body cells damaged during tissue preparation. It must be assumed that the bands seen are due to proteins which have been exported while the fat body tissue has been in the culture medium.

It might have been expected that the stain intensity may have increased for the samples that had been in the tissue culture medium for a longer period of time; Figure 5.23 shows that this was not the case and that proteins were released from the fat body cells as soon as the were placed in the tissue culture medium and that this export did not continue if the tissue remained in the medium for a longer time. This may be due to the unsuitability of the tissue culture medium used or that the fat body cells ceased to export protein due to the loss of a particular signal after removal from the insect.

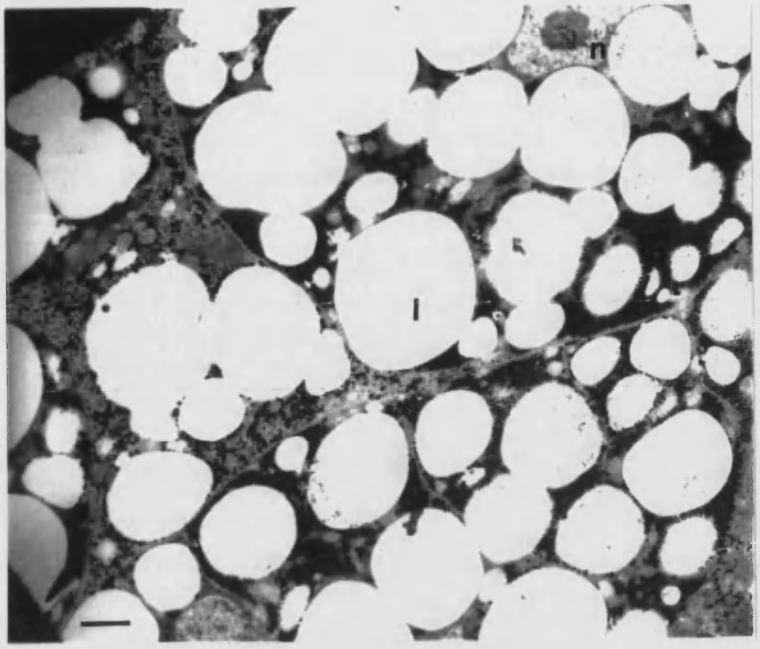


Figure 5.1 *M. speyeri* embryo larva fat body cells (Bar = 2 μm).

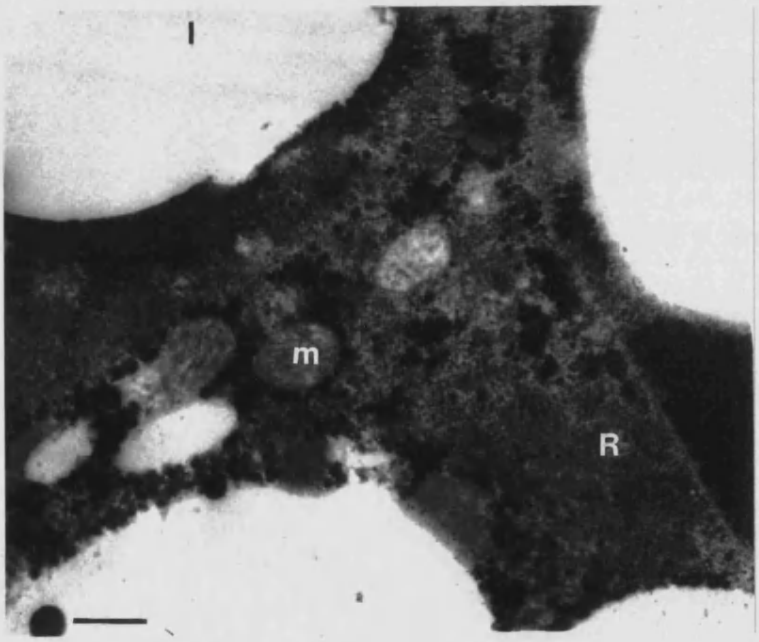


Figure 5.2 *M. speyeri* embryo fat body cell cytoplasm (Bar = 500 nm).

(Key l = lipid droplet, n = nucleus, m = mitochondria, R = rough endoplasmic reticulum).

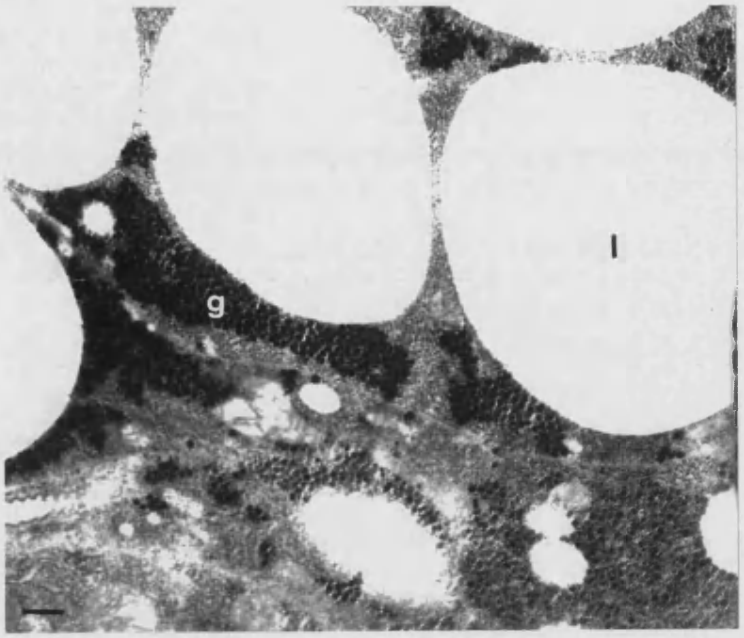


Figure 5.3 *M. speyeri* embryo fat body cell cytoplasm (Bar = 500 nm).

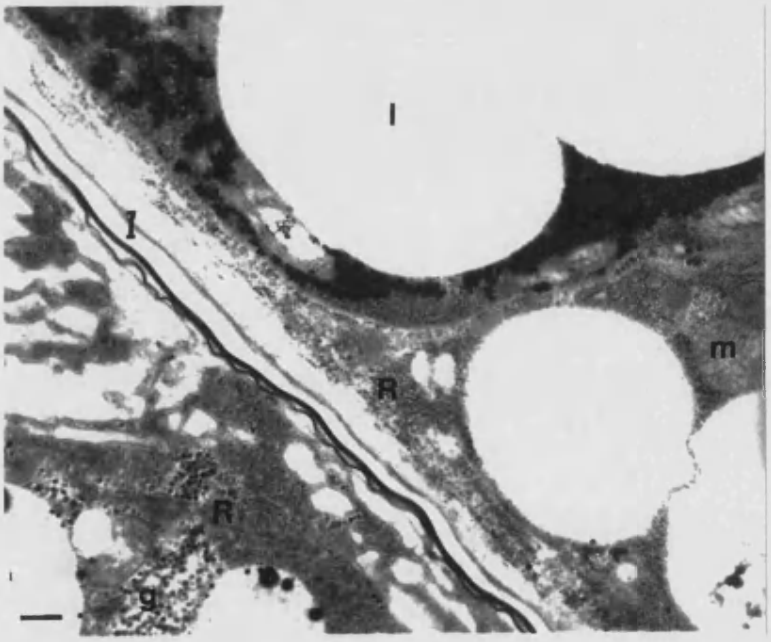


Figure 5.4 *M. speyeri* embryo fat body cell cytoplasm, with embryo maternal interface (Bar = 500 nm) (Key l = lipid droplet, n = nucleus, m = mitochondria, R = rough endoplasmic reticulum, g = glycogen, I = embryo maternal interface).

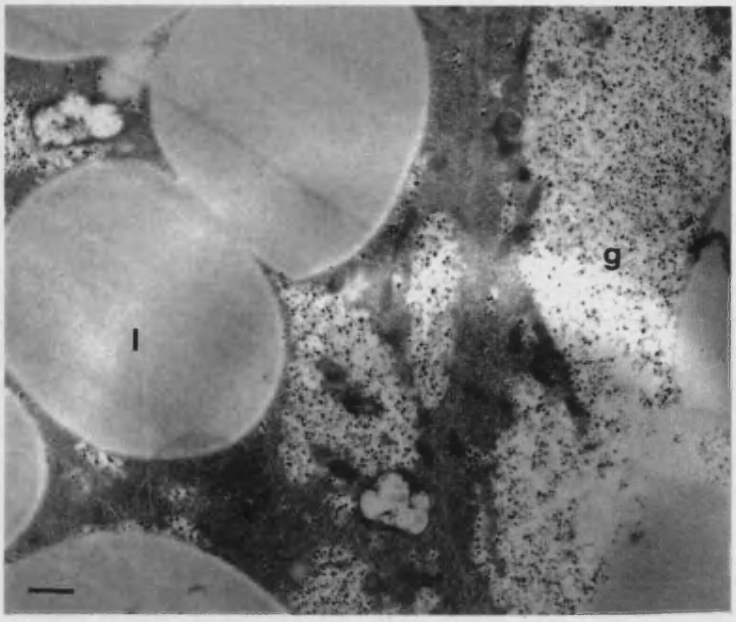


Figure 5.5 One day old, *M. speyeri* larval fat body cells (Bar = 1 μm).

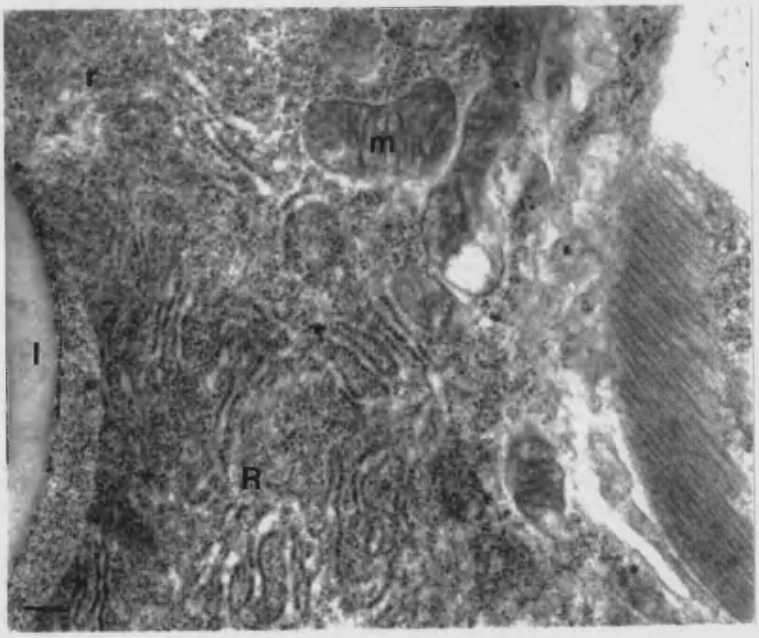


Figure 5.6 One day old, *M. speyeri* larval fat body cells (Bar =200 nm).

(Key l = lipid droplet, m = mitochondria, R = rough endoplasmic reticulum, g = glycogen, c = cytoplasm, r = ribosome).

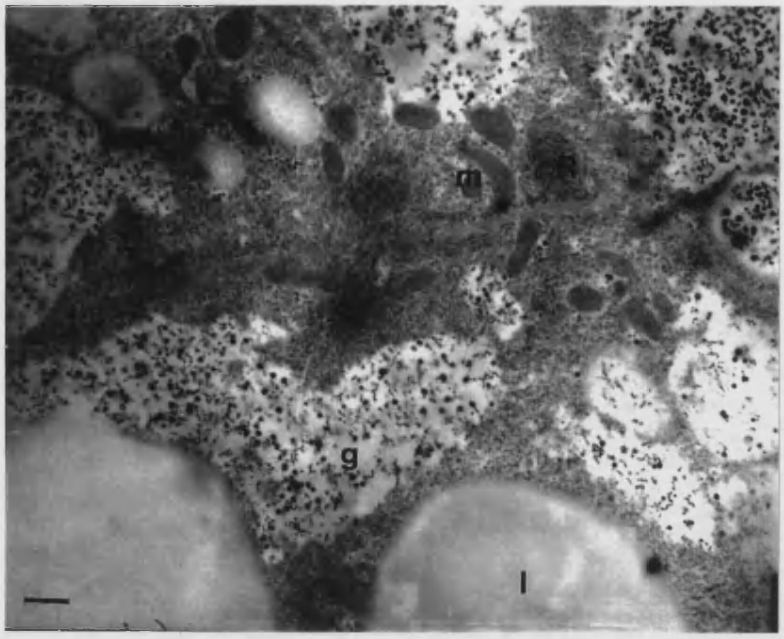


Figure 5.7 One day old, *M. speyeri* larval fat body cells (Bar = 500 nm).

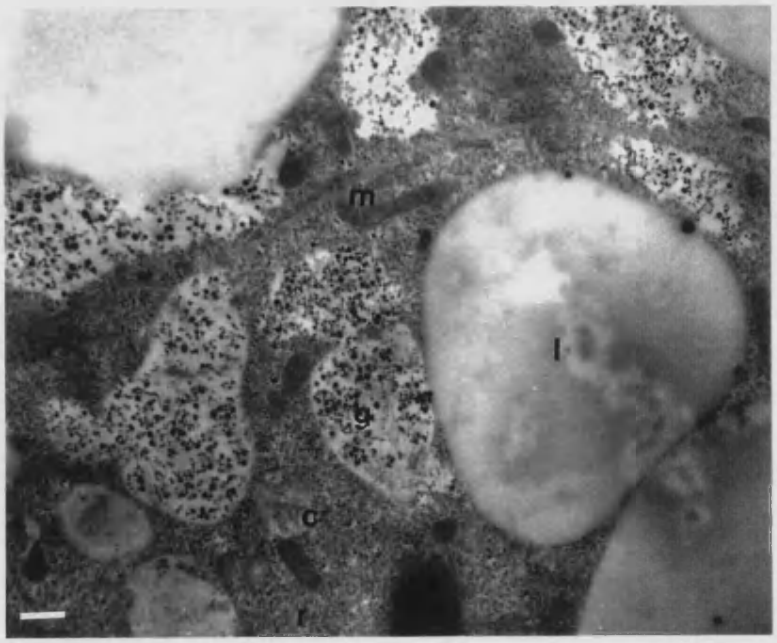


Figure 5.8 One day old, *M. speyeri* larval fat body cell (Bar = 500 nm).

(Key l = lipid droplet, m = mitochondria, R = rough endoplasmic reticulum, g = glycogen, c = cytoplasm, r = ribosome).

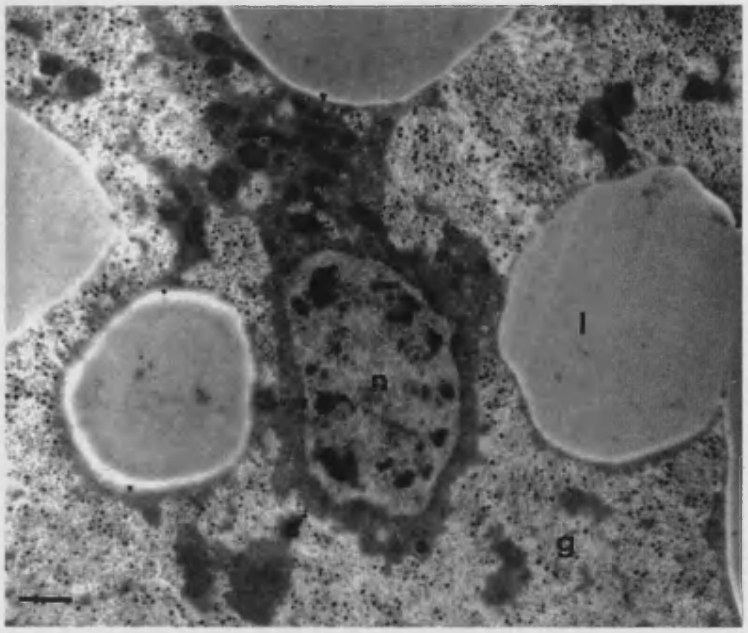


Figure 5.9 Three day old, *M. speyeri* larval fat body cell (Bar = 1 μm).

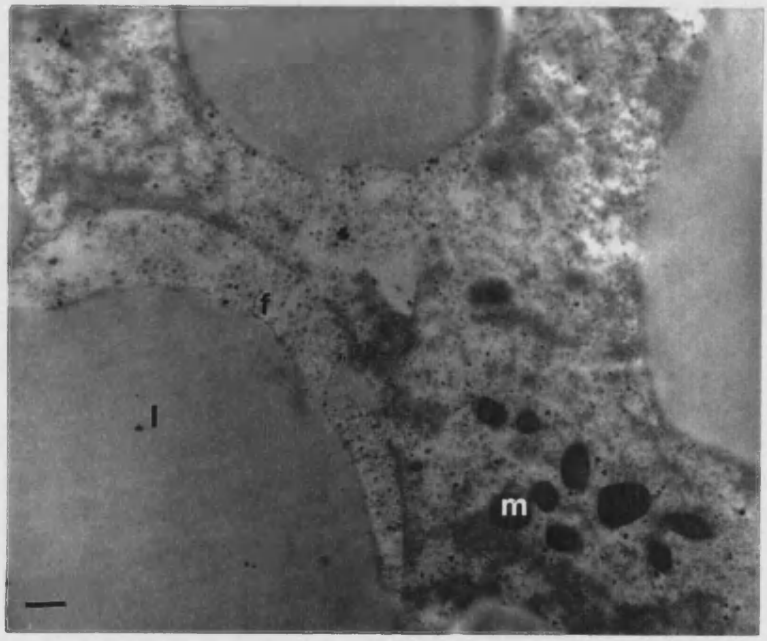


Figure 5.10 Three day old, *M. speyeri* larval fat body cell cytoplasm (Bar = 500 nm). (Key l = lipid droplet, m = mitochondria, g = glycogen, n = nucleus, f = filled lipid droplet).

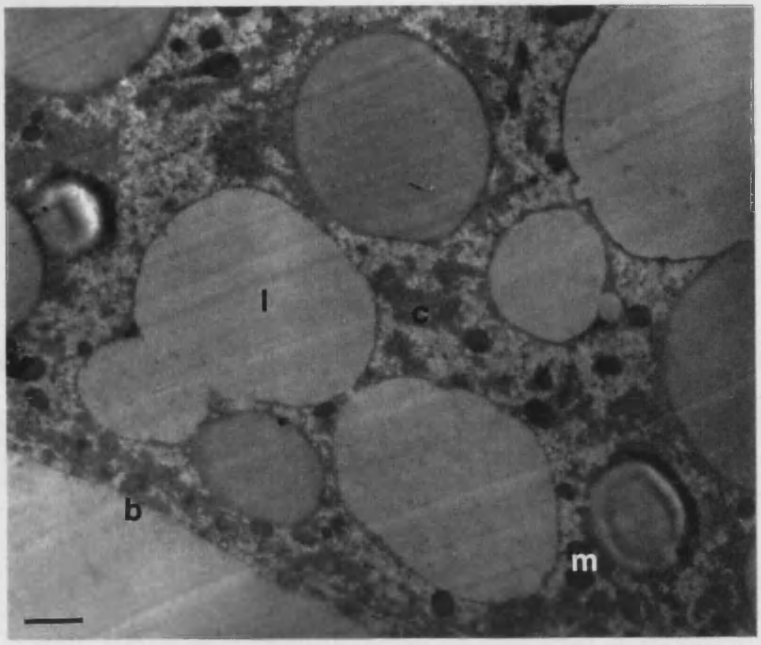


Figure 5.11 Three day old, *M. speyeri* larval fat body cell cytoplasm (Bar = 1 μm).

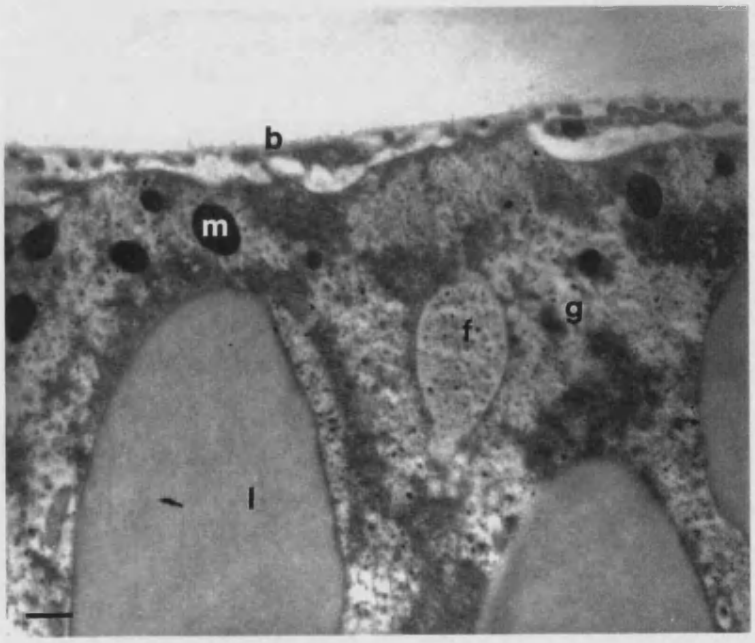


Figure 5.12 Three day old, *M. speyeri* larval fat body cell cytoplasm (Bar = 500 nm).

(Key l = lipid droplet, m = mitochondria, g = glycogen, c = cytoplasm, b = boundary, f = filled lipid droplet).

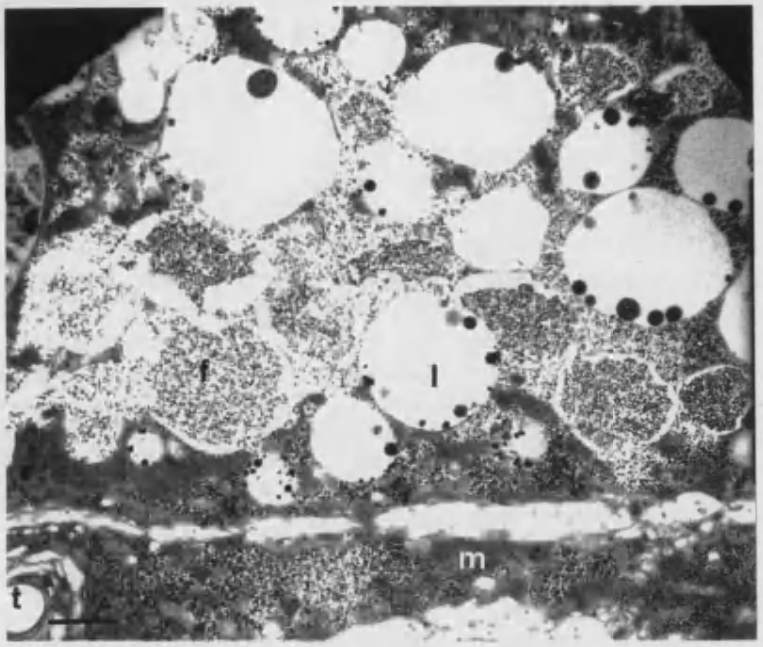


Figure 5.13 Five day old, *M. speyeri* larval fat body cells (Bar = 2 μm).

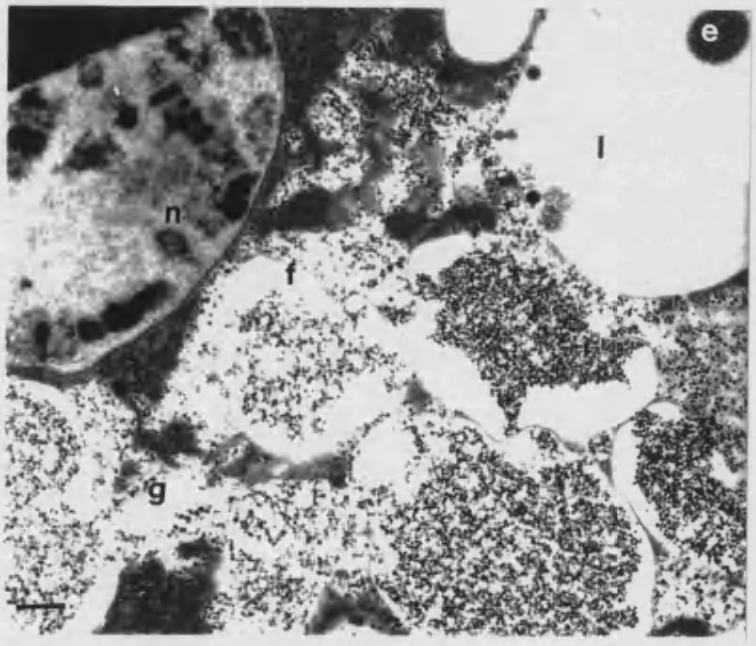


Figure 5.14 Five day old, *M. speyeri* larval fat body cell cytoplasm (Bar = 1 μm).

(Key l = lipid droplet, m = mitochondria, t = trachea, f = filled lipid droplet, e = electron dense body, g = glycogen, n= nucleus).

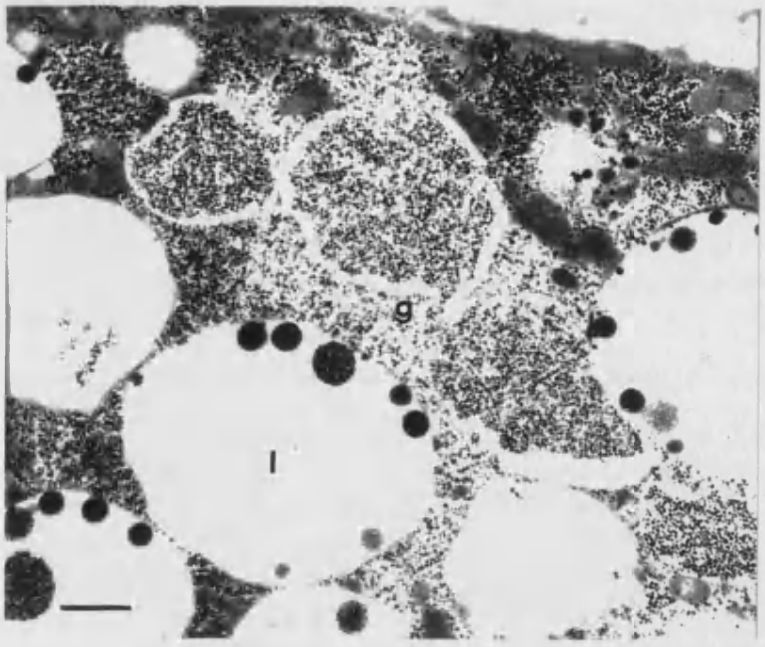


Figure 5.15 Five day old, *M. speyeri* larval fat body cell cytoplasm (Bar = 1μm).

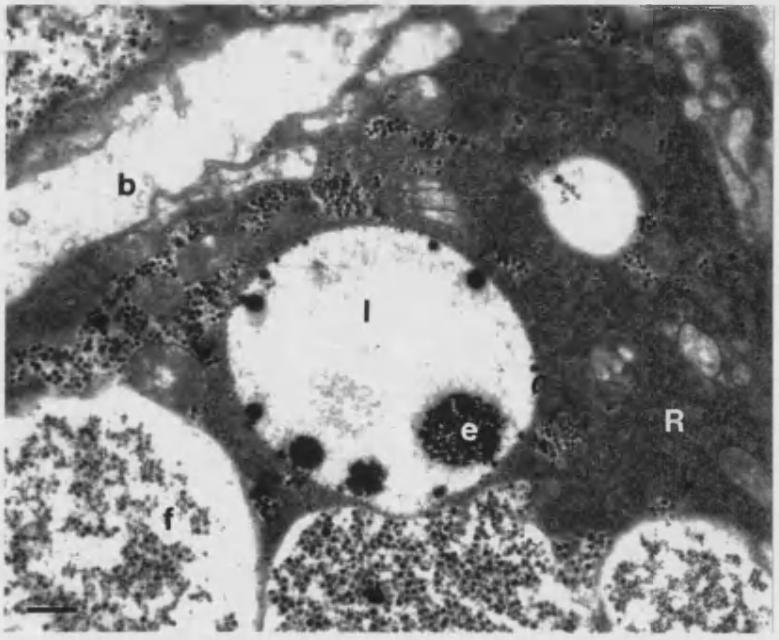


Figure 5.16 Five day old, *M. speyeri* larval fat body cell cytoplasm (Bar = 500 nm).

(Key l = lipid droplet, g = glycogen, e = electron dense body, f = filled in lipid droplet, R = rough endoplasmic reticulum, b = cell boundary).

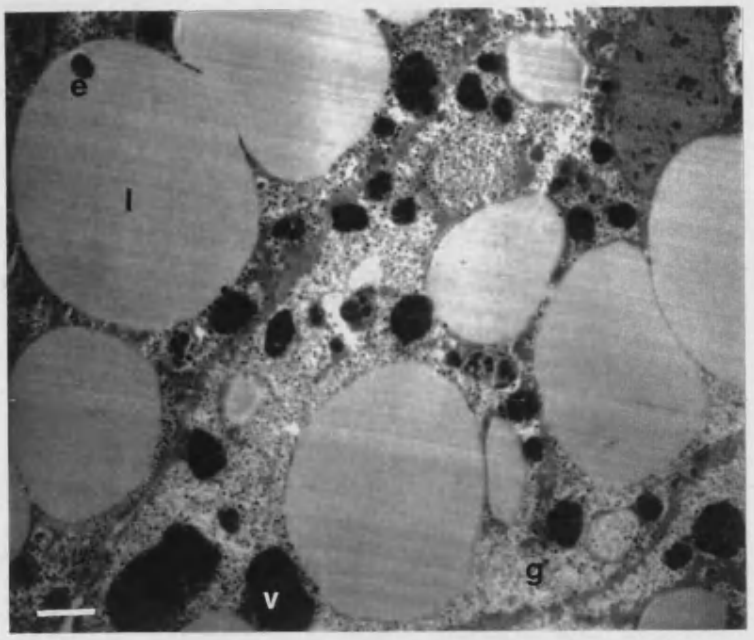


Figure 5.17 *M. speyeri* pupal fat body cells (Bar = 2 μm).

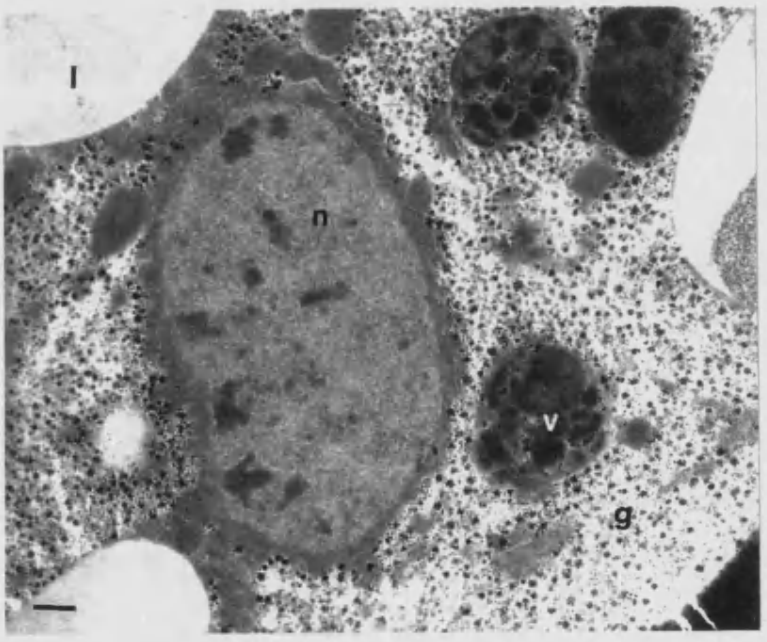


Figure 5.18 *M. speyeri* pupal fat body cell cytoplasm (Bar = 500 nm).

(Key l = lipid droplet, n = nucleus, g = glycogen, e = electron dense body, v = multivesicular body).

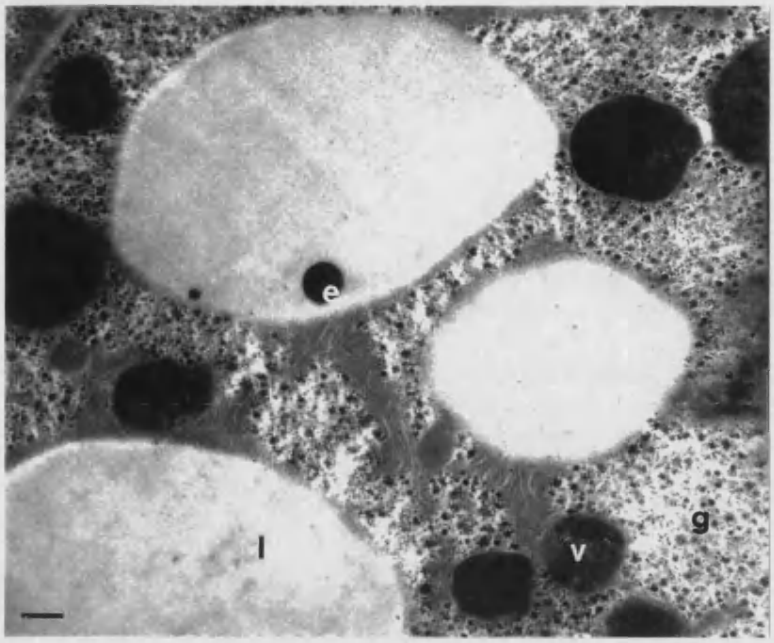


Figure 5.19 *M. speyeri* pupal fat body cell cytoplasm (Bar = 500 nm)

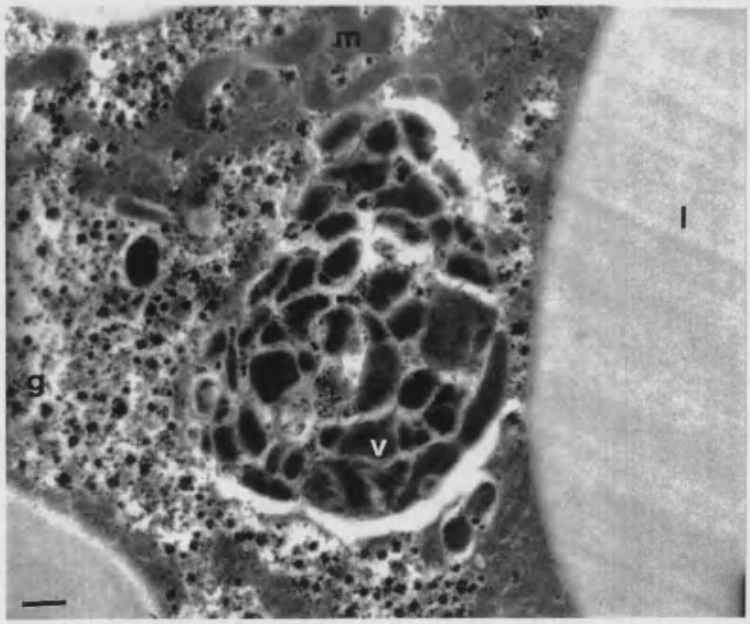
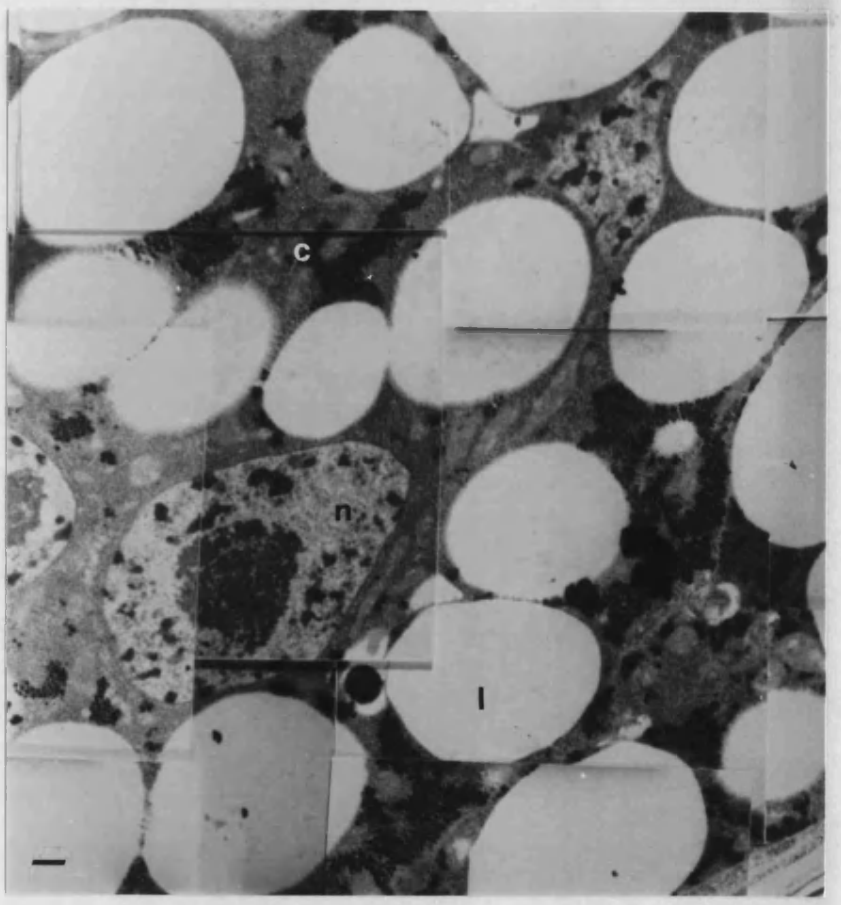
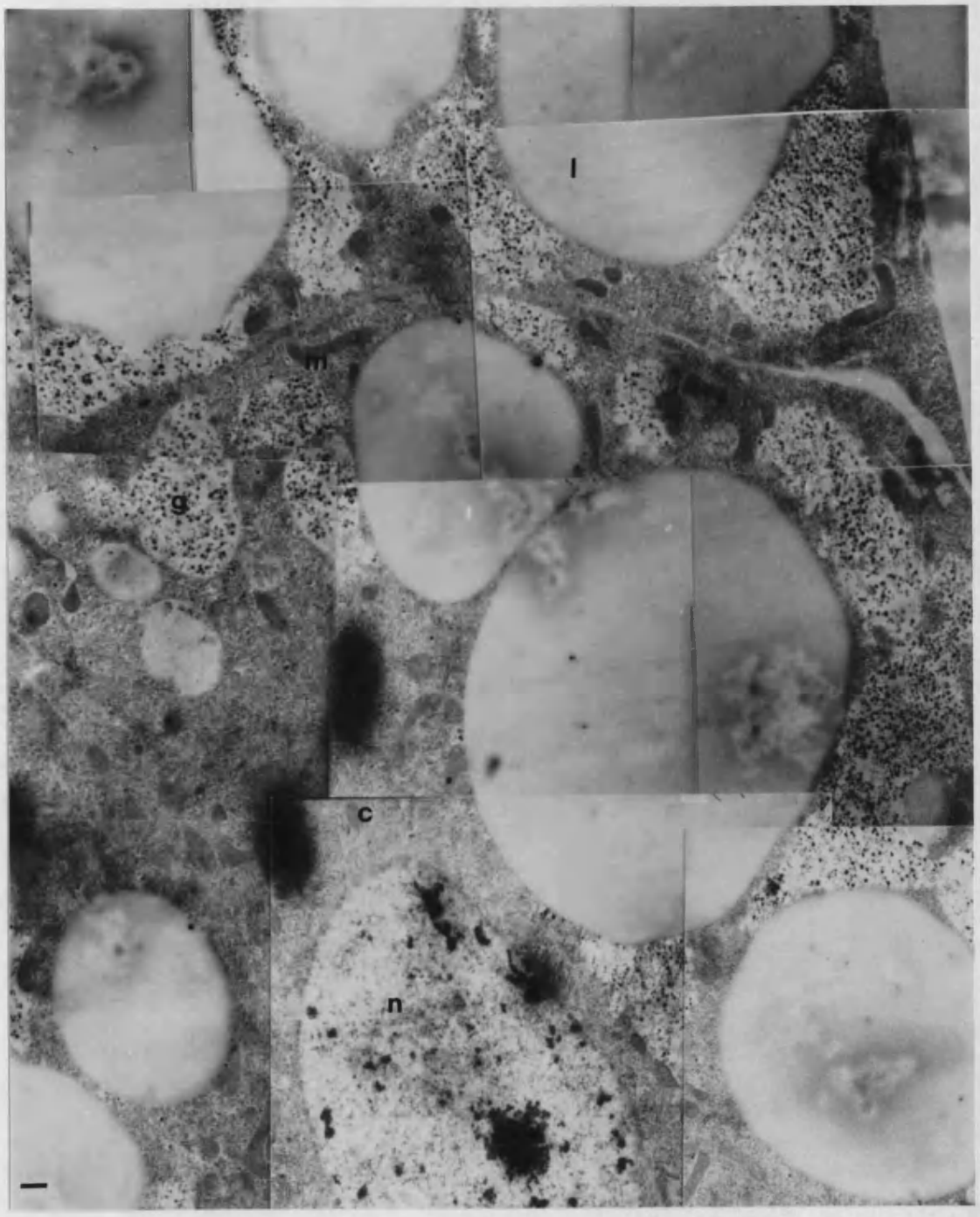


Figure 5.20 Multivesicular body found in *M. speyeri* pupal fat body cell cytoplasm (Bar = 500 nm)(Key l = lipid droplet, v = multivesicular body, m = mitochondria, g = glycogen, e = electron dense body)



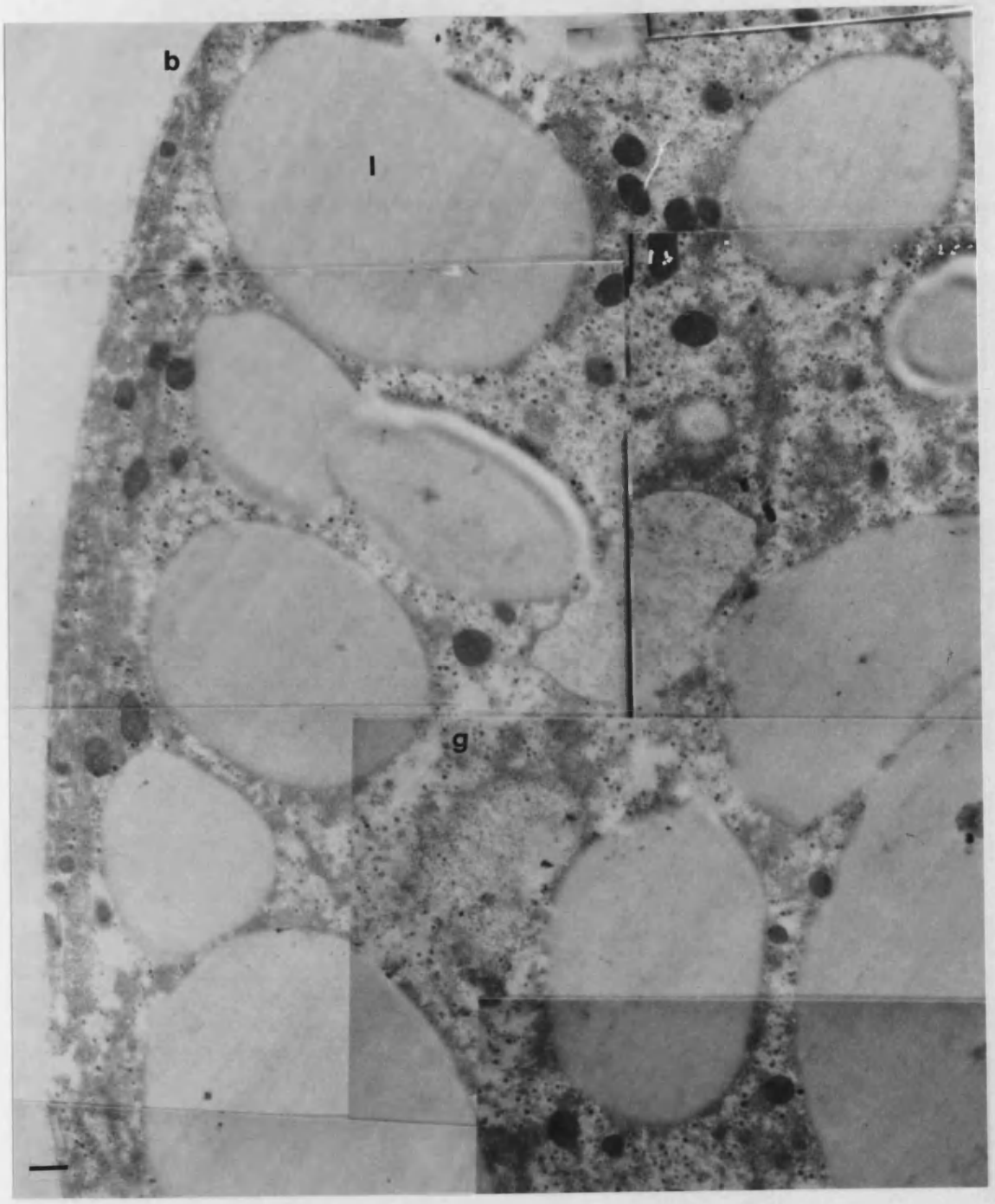
Montage 1 *M. speyeri* embryo fat body tissue (Bar = 500 nm).

(Key l = lipid droplet, n = nucleus, g = glycogen, c = cytoplasm)



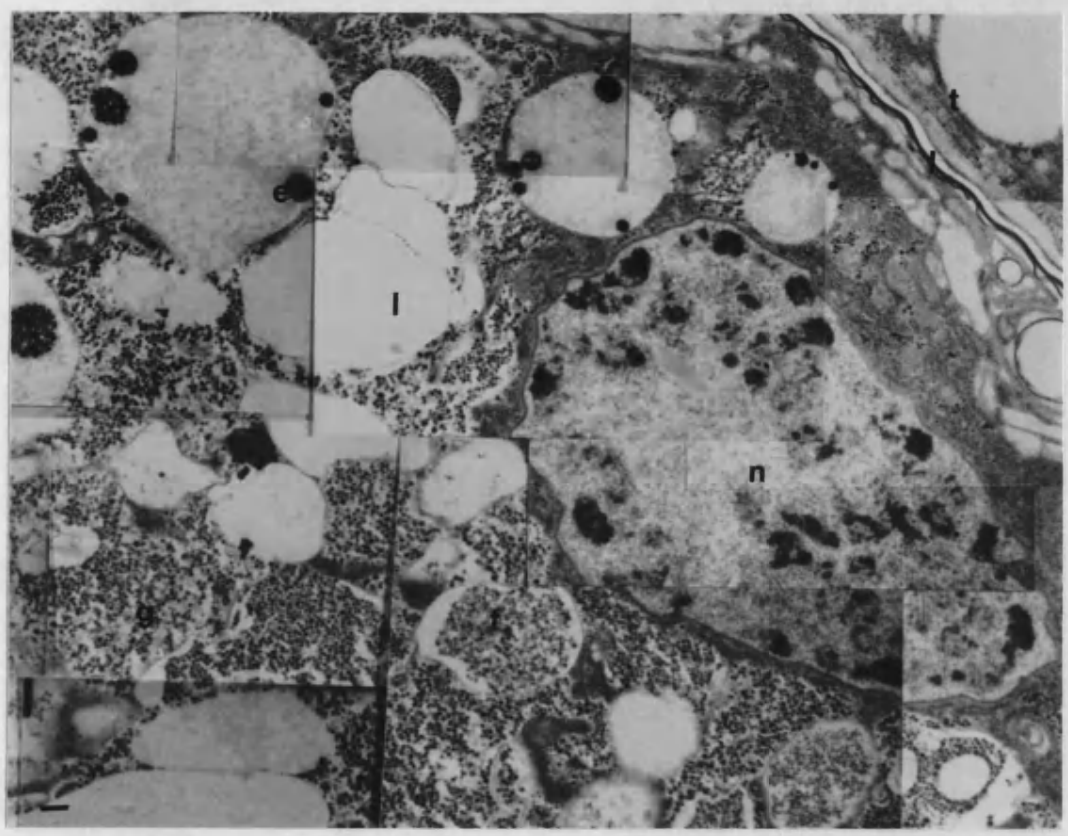
Montage 2 One day old *M. speyeri* larval fat body tissue (Bar = 500 nm).

(Key l = lipid droplet, m = mitochondria, n = nucleus, c = cytoplasm, g = glycogen).



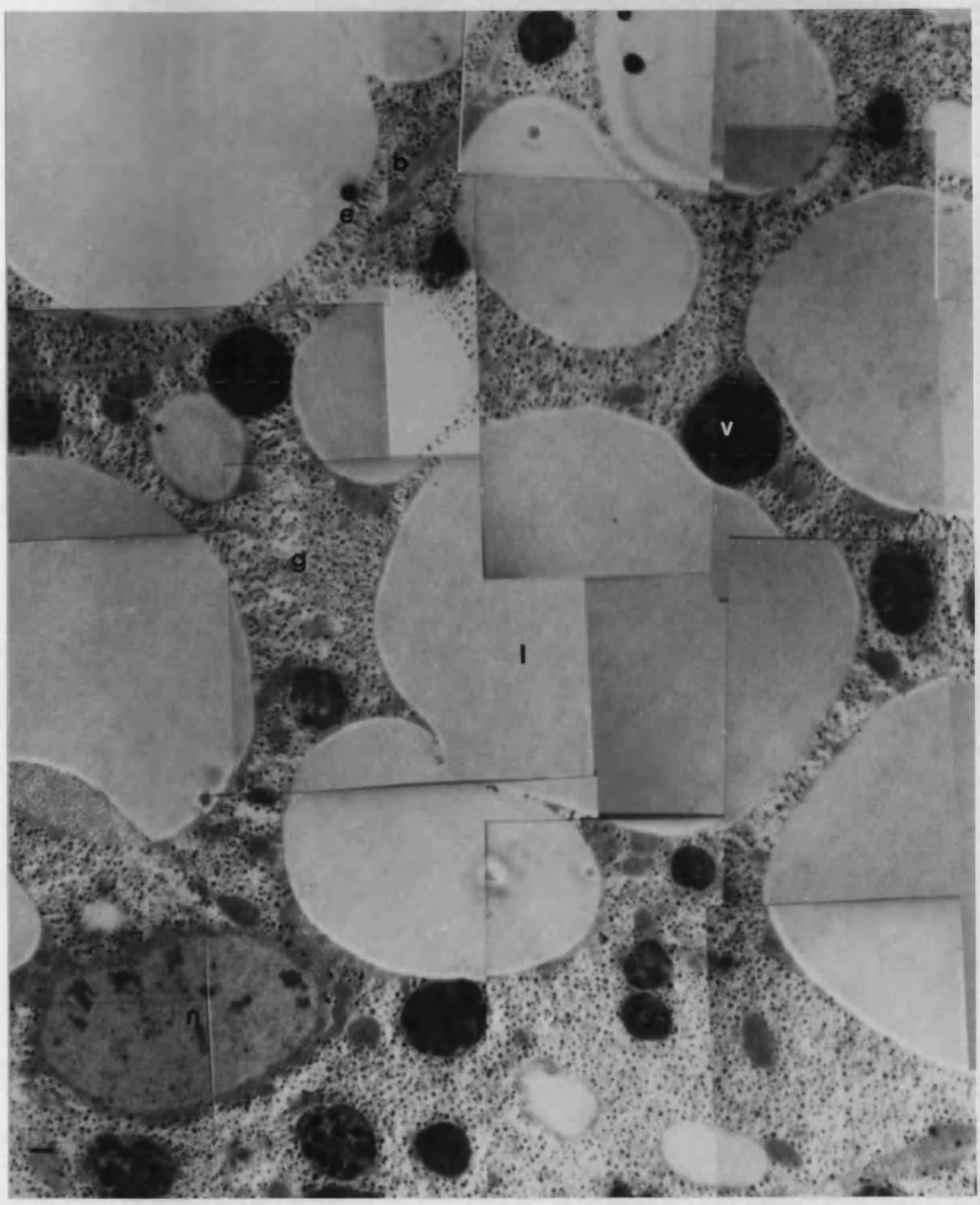
Montage 3 Three day old, *M. speyeri* larval fat body tissue (Bar = 500 nm).

(Key l = lipid droplet, g = glycogen, m = mitochondria, b = cell boundary).



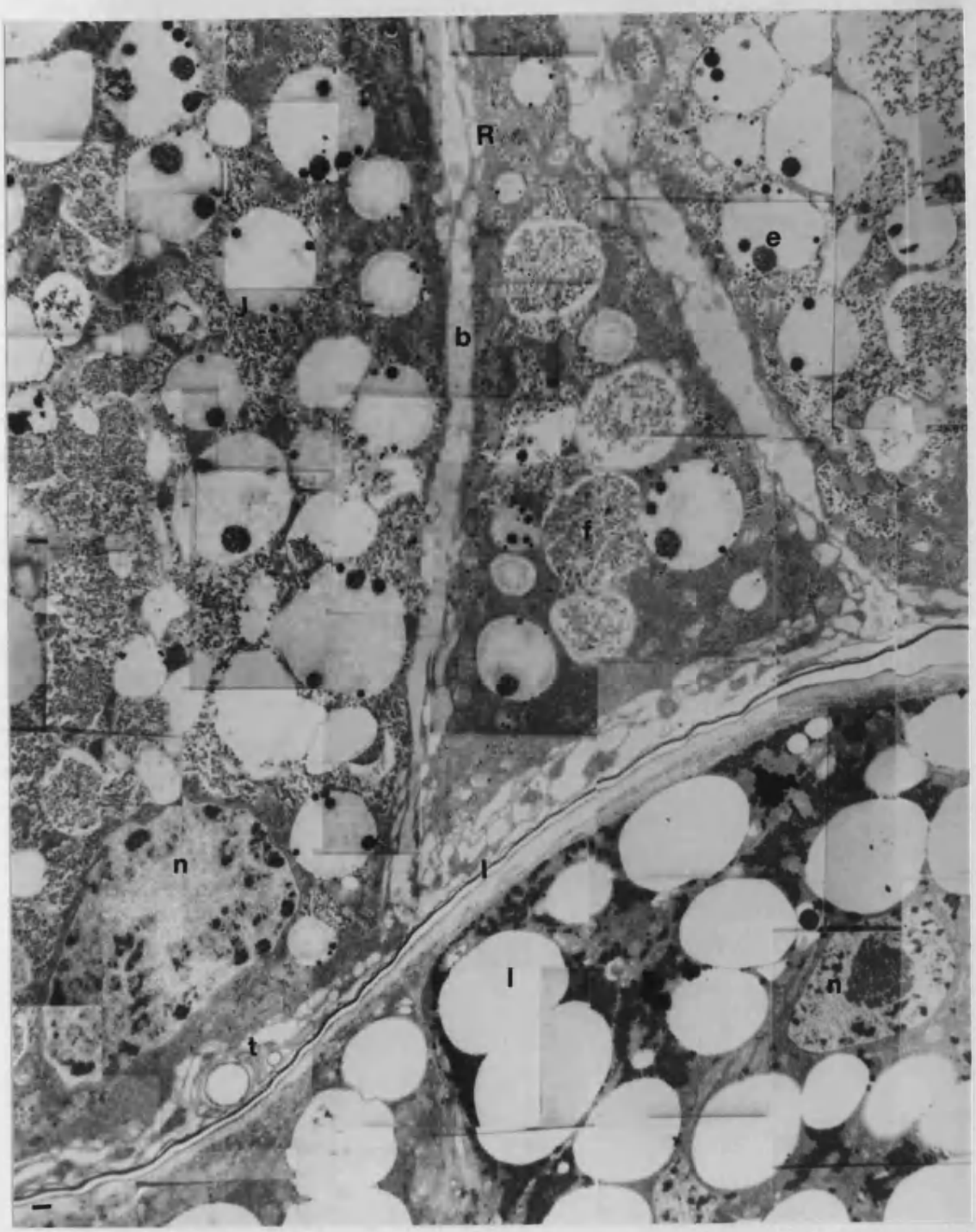
Montage 4 Five day old *M. speyeri* larval fat body tissue (Bar = 500 nm).

(Key l = lipid droplet, n = nucleus, e = electron dense body, t = trachea, I = embryo/maternal interface, g = glycogen, f = filled lipid droplet).



Montage 5 *M. speyeri* pupal fat body tissue (Bar = 500 nm).

(Key l = lipid droplet, n = nucleus, v = multivesicular body, g = glycogen, e = electron dense body, b = cell boundary).



Montage 6 *M. speyeri* fat body tissue. Interface between maternal fat body cells and embryo fat body tissue (Bar = 500 nm).

(Key l = lipid droplet, n = nucleus, R = rough endoplasmic reticulum, g = glycogen, e = electron dense body, t = trachea, I = embryo/maternal interface, f = filled lipid droplets, b = cell boundary).

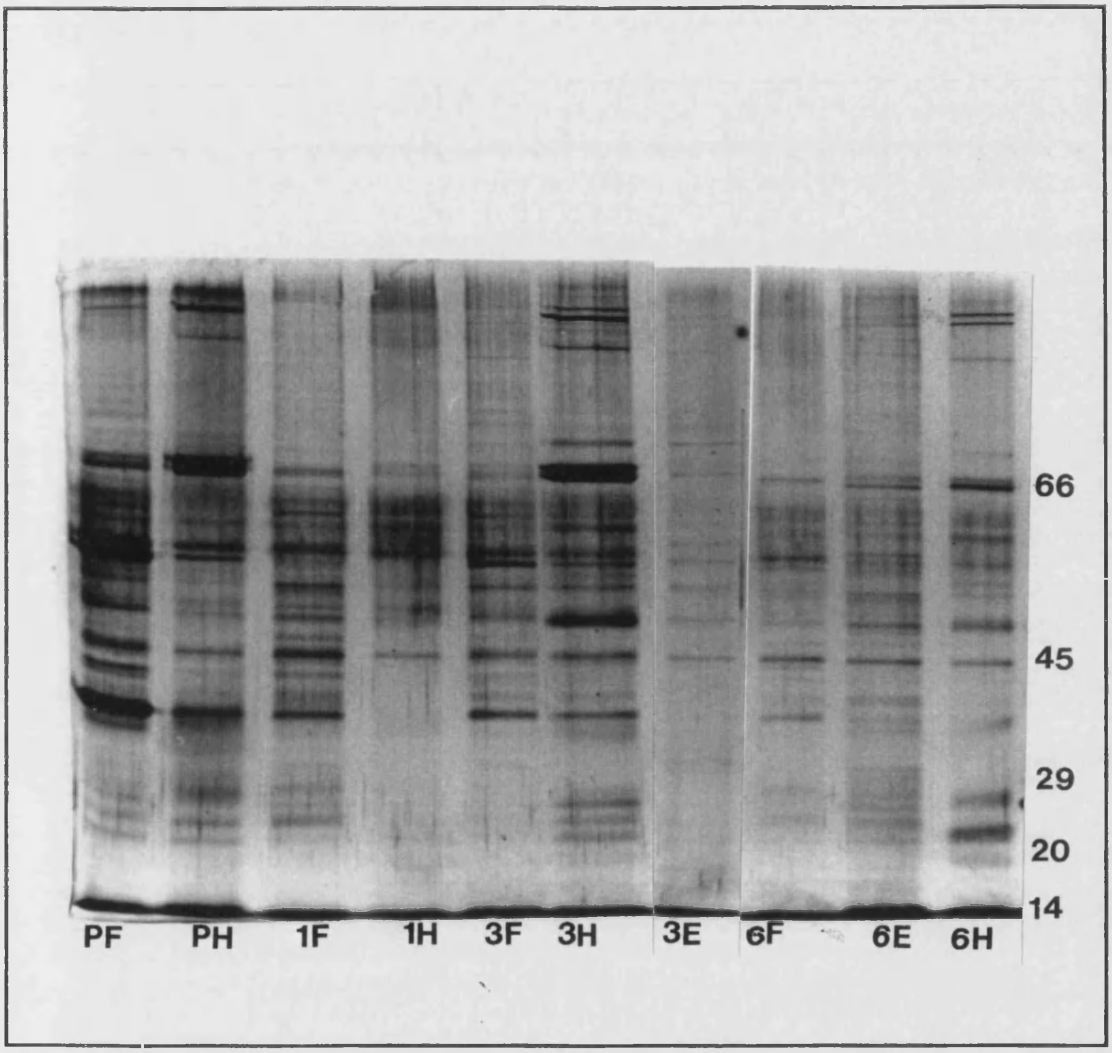


Figure 5.21 Silver stained SDS-PAGE gel of *M. speyeri* tissue samples.

1H: 1 day haemolymph, 1F: 1 day fat body, 3H: 3 day haemolymph, 3F: 3 day fat body, 3E: 3 day embryo, 6H: 6 day haemolymph, 6F: 6 day fat body, 6E: 6 day embryos, PH: pupal haemolymph, PF: pupal fat body.

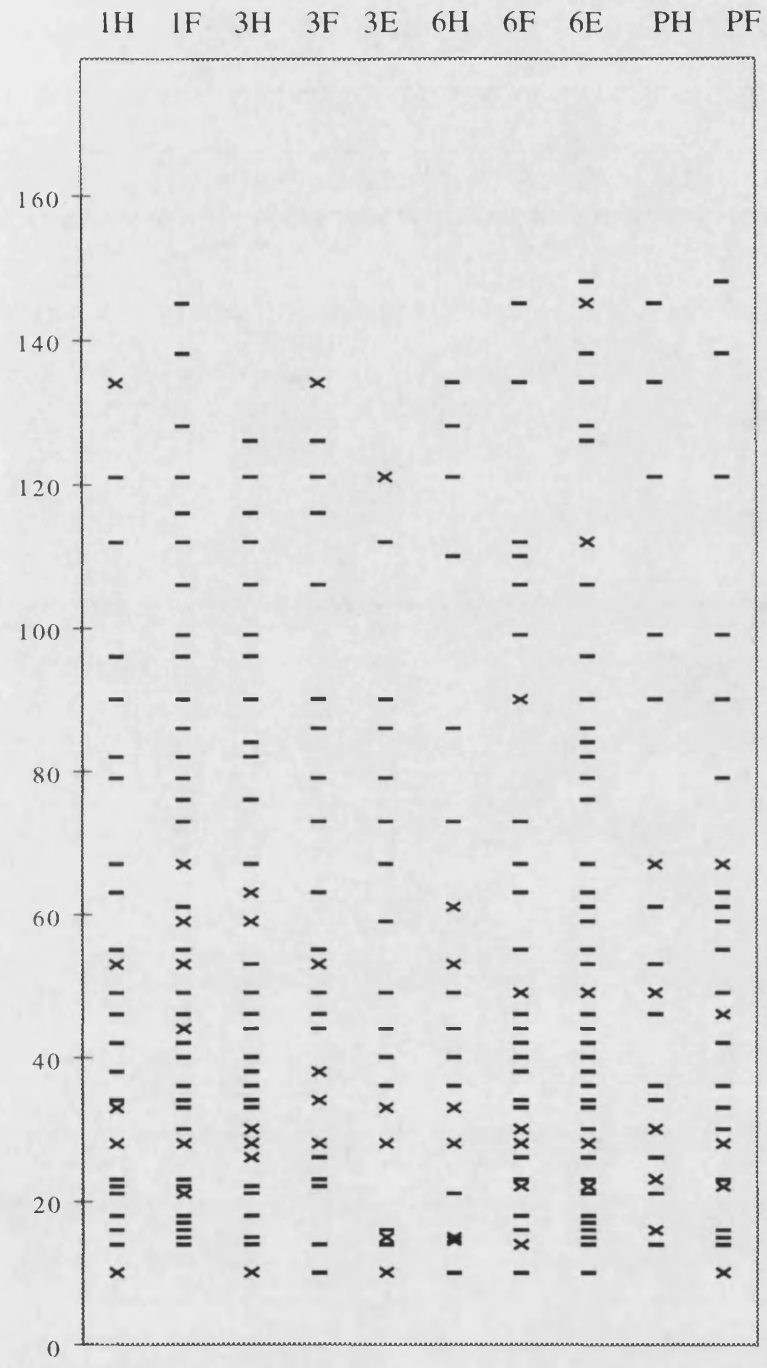


Figure 5.22 Diagrammatic representation of SDS-PAGE gel of *M. speyeri* samples showing the molecular weight of each band.

Bands represented by the symbol × are the proteins most strongly expressed in each tissue sample.

1H: 1 day haemolymph, 1F: 1 day fat body, 3H: 3 day haemolymph, 3F: 3 day fat body, 3E: 3 day embryo, 6H: 6 day haemolymph, 6F: 6 day fat body, 6E: 6 day embryos, PH: pupal haemolymph, PF: pupal fat body.

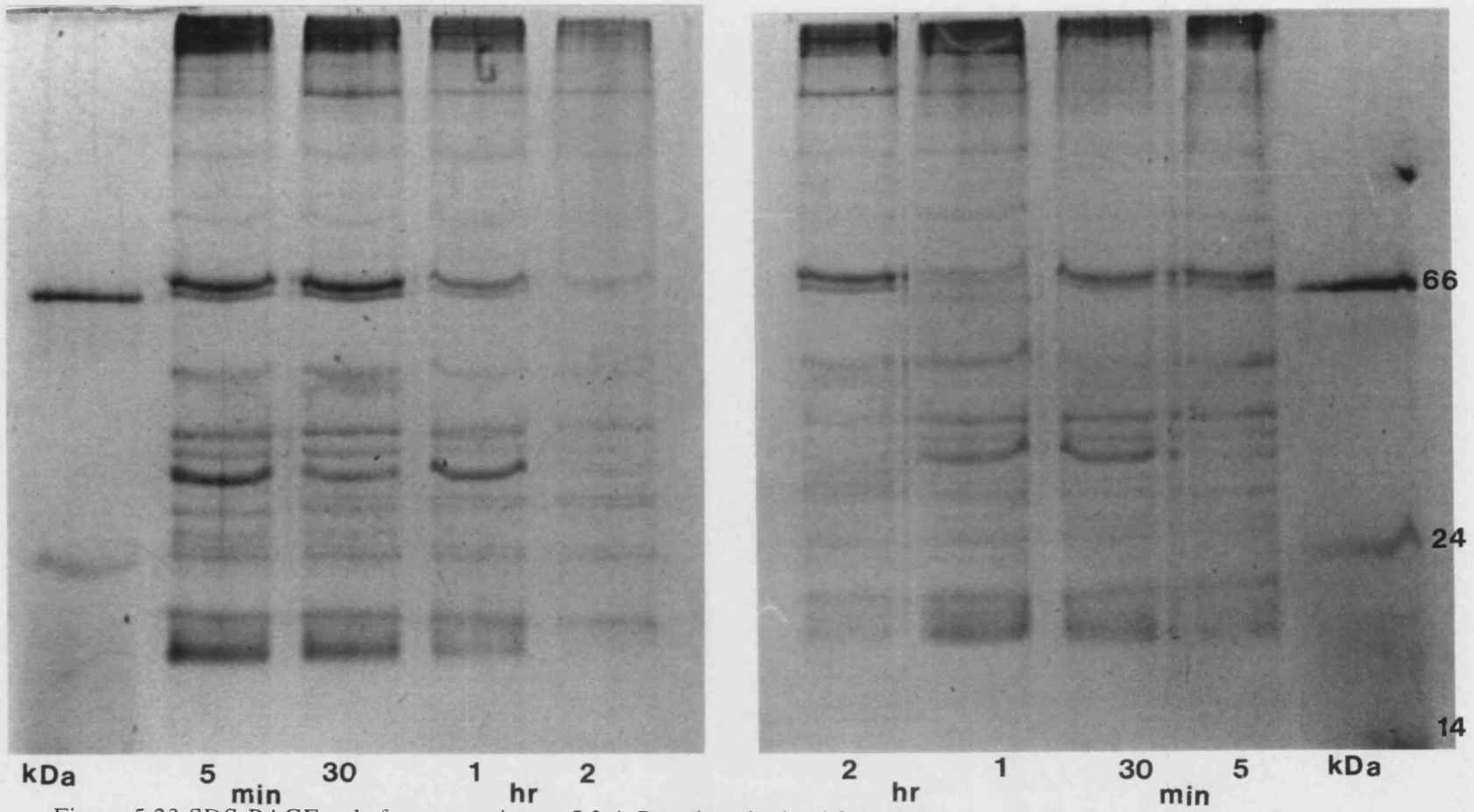


Figure 5.23 SDS-PAGE gels from experiment 5.2.4. Proteins obtained from *M. speyeri* fat body cells during *in vitro* culture.

5.4 Discussion.

Cecid larvae were fixed and prepared for TEM using a method adapted from Meats and Tucker (1976). The tissue was viewed and the structure and cell processes within the fat body were studied.

A method was developed to dissect paedogenetic larvae and to separate the tissues of the fat body, the haemolymph and the embryos. The protein was extracted from these tissues and quantified. The proteins were then separated using SDS- PAGE and the gels were stained to show the proteins using silver staining technique. A method was also developed to culture fat body cells from paedogenetic larvae *in vitro*. The proteins that the cells produced while in culture were separated using SDS- PAGE and the gels were also stained to show the proteins using silver staining technique.

During the experiments described in this chapter particular attention was paid to the cellular processes occurring within the paedogenetic fat body cells and the proteins contained within the tissues. This was in order to ascertain how the developing embryos were nourished during this unusual form of reproduction.

From previous studies made by Went and Camenzind (1980) and Ivanova-Kasas (1965) it was clear that the eggs produced precociously by the paedogenetic larvae were dissimilar to those produced by oviparous insects in that they do not contain significant amounts of yolk reserves for the developing embryos. It is also true to say that the embryonic larvae cannot obtain nutrition by physically consuming the mother

because they are contained within the follicular epithelium and they do not posses mouthparts designed for biting and chewing.

There must be a method by which the maternal tissues produce proteins which are then accumulated by the growing larva through the course of development. It cannot be the case that the embryonic larva simply absorbs nutrients from the food ingested by the mother as without a fully developed digestive system as it would be unlikely that they could utilise the fungal protoplasts as ingested by the mother.

Some authors such as Ivanova-Kasas (1965) suggest that the larvae act as parasites and feed off the maternal tissues. Ivanova-Kasas (1965) even suggests that the larvae exert enzymatic action on the maternal tissues to induce their breakdown. Although it can be seen, when studying these insects, that on emerging the offspring larvae leave little of the mother behind them except the tracheae and the cuticle, it is clear from the TEM pictures of the 6 day old larval fat body that this is not a degenerating tissue. The fat body cells still have intact nuclei, mitochondria and nutrient reserves and show no evidence of being in contact with degrading enzymes.

This all suggests that the fat body is a tissue which actively provides nutrients for the offspring, not one which is parasitised by them. The fact that post offspring emergence there is little left of the mother reflects the completeness of the nutrient transfer. There were no signs of cell death in the maternal fat body, even in 6 day old larvae. The final disintegration of the fat body may be explained by the mechanical damage caused by

up to 30 fully grown offspring larvae moving within the confined space of the cuticle against the remains of the fat body which is a delicate tissue.

The ultrastructural study undertaken highlights the role of the cecid fat body as a tissue which stores nutrients. One of these stored nutrients is lipid. The fat body from all four stages appeared to contain lipid droplets. Many of these droplets disappeared through the course of development and were replaced by cytoplasm.

There was also some evidence to suggest that the lipid contained within these droplets was conjugated with proteins which were produced within the fat body. There appeared to be empty lipid vesicles in the embryo and day 5 insects, it could be that they were empty because they contained only lipid which was dissolved away during preparation. The other stages appeared to have contents; this may have been the protein left after the lipid had been dissolved.

This would explain why there did not seem to be any stored protein, even though there was ample evidence of protein production; it was being stored within the lipid droplets. There was no protein within the lipid droplets of the embryonic tissue because at this stage none had been made. It may be suggested that the fat body synthesises protein during the period of rapid embryonic growth, and that this is stored in the lipid droplets before being moved out of the cells to provide nutrients for the paedogenetic offspring during days 1 - 4. By day 5 much of the lipid and protein has apparently left the cell, leaving many empty vesicles which are filled with cytoplasm containing many

glycogen granules and many vesicles which only contain lipid which, after processing, appear empty.

This would also explain why the ribosome-type cytoplasm and the RER is often closely associated with the lipid droplets. Other authors have also suggested that proteins within the fat body cells may be associated with lipids (Price 1973). It would also offer an explanation as to why the fat body loses its orange colouration at the same time that the embryo larvae take on their orange colour.

Although there was no evidence of protein storage, much glycogen was seen to be stored in the form of granules within the cytoplasm. Glycogen is a carbohydrate and like lipids provides only stored energy. This would be needed not only for respiration, but also for the synthesis of chitin, a major constituent of the insect's cuticle.

The cytoplasm also contained mitochondria and much evidence of protein production. (RER, ribosomes). There was no evidence of the protein produced within the fat body cells being exported to the embryos or the haemolymph, even though the cell boundaries were studied extensively . There was a small amount of damage at the boundaries of some cells but this may have been due to mechanical damage during tissue preparation. There was no evidence seen of the fat body being used to store waste products such as uric acid.

The stages of development seen in the *M. speyeri* larval fat body studied were similar to those described by Raikhel and Lea (1983) and Raikhel (1987) for adult mosquito

fat body during vitellogenesis, except for the termination phase seen in the mosquito during which there were signs of cell death.

In the cecids the process of embryonic nutrition differs from the vitellogenic process seen in other insects because the nutrients produced by the fat body are not laid down as yolk in the egg, but are instead provided for use by the developing embryos. The lack of fat body cell death in cecids after this period of production may be due to the fact that these insects are, at this stage, in an immature form and would, under normal circumstances, still be expected to produce adult insects.

Polyacrylamide gel electrophoresis (Figure 5.19) was used to separate the proteins contained within the tissues of *M. speyeri*. It shows that there are many proteins produced by the fat body, many of these can also be found in the haemolymph and many of the bands seen in the sample taken from the fat body also appear in the embryonic samples. This suggests that the fat body does provide nutrients for the embryo.

However, there is not a single protein, or a small number of proteins, that dominate the gels of Figure 5.19, indicating that protein transfer from maternal fat body to the developing embryo inVolumeves several or many proteins. It is not clear whether the cecid fat body synthesises special transfer proteins resembling (or even homologous with) the vitellogenins of other insects. The haemolymph of 3 day old larvae has a particularly prominent pair of bands at around 66 kDa. A great deal of further work would be required to say whether this protein was a vitellogenin precursor or subunit.

The work carried out on proteins exported by fat body cells cultured *in vitro,* showed that some proteins were exported from the fat body cells into the tissue culture medium. These proteins were found to be identical to those obtained from samples of haemolymph taken from 3 day old larvae. They did not match the proteins obtained from a homogenate of fat body cells. This shows that the fat body cells are responsible for the export of proteins into the haemolymph; it is not clear whether any of these are vitellogenic in origin or what their role in larval nutrition may be.

The method by which haemolymph proteins and other nutrients are taken up by the developing embryos is unknown. The presence of an embryonic cuticle surrounding the embryo would seem to pose a problem for a hypothetical uptake mechanism that involved absorption of nutrients at the surface of the embryo by the embryonic epithelial cells. However, it should be pointed out that the embryonic cuticle might be relatively permeable to dissolved solutes, and might therefore pose little barrier to absorption. There are precedents for cuticle with such properties. For example, the mid-gut of most insects is linked with a cuticle-like peritrophic membrane (Spence 1991).

Moreover, the cuticle lining of the hindgut has been directly shown to be permeable to dissolved nutrients (Maddrell and Gardiner 1980). Some controversy exists on the question of whether the products of the digestion of the old cuticle are directly absorbed through the new cuticle during moulting, but this has not yet been disproved,

and a number of authors assert that this does occur (reviewed by Reynolds and Samuels 1996).

If absorption at the surface of the embryo does occur, then evidence of the process in the form of coated vesicles would have been expected to be seen in the developing embryos. This would be like the process of protein sequestration that is known to occur in last-instar lepidopteran fat body (Locke and Collins 1968), and also in the developing ova of other insects (e.g. Telfer and Pan 1988). However , it should be remembered that only in day 5 paedogenetic larvae was it possible to examine embryos *in situ*. These embryos were very mature and it is possible that nutrient uptake might already have ceased.

In summary: it is clear that the fat body does produce proteins which are utilised by the growing embryos. Ultrastructural studies suggest that the embryos do not obtain these nutrients by attacking or causing the degeneration of the maternal tissue but by accumulating the nutrients from the surrounding haemolymph.

Chapter 6

General Discussion.

Some mycetophagous species of the Cecidomyiidae have the ability to reproduce paedogenetically. This type of parthogenetic, asexual reproduction, which occurs while these insects are still at the larval stage, allows the rapid build up of populations.

The ability to colonise new areas very rapidly in this way has made certain species of cecids very serious pests of cultivated mushrooms (Flegg *et al.* 1985, Fletcher *et al.* 1989). This high value crop can be ruined if mushroom houses become infested. The insects can depress yields of the crop, spread diseases within the mushroom house and their presence causes cosmetic damage making the mushrooms unsaleable. Cecids have proved to be a difficult pest to control in the field. They appear to be very resistant to many pesticides (White 1986) and the control of larval populations is pointless as once they have become established as their presence will have already reduced the commercial value of the crop. The present work had aimed to increase the knowledge of cecid reproductive biology with the strategic aim of preventing the build up of cecid populations in cultivated mushrooms.

A number of authors document the processes which occur during paedogenetic reproduction (e.g. Wagner 1863, Camenzind 1962, Matuszewski 1982, Went 1982 and Mamaev and Krivosheina 1993). Many of these accounts are contradictory with much

confusion existing over subjects such as the origins of the nurse cells produced during oogenesis.

The process of paedogenesis is an extraordinary method of reproduction during which eggs develop parthogenetically within the haemocoel of another larva. These eggs grow at the expense of the maternal tissues and after around 5 days the resulting larvae emerge fully developed from the maternal cuticle already containing developing eggs of the next generation.

It was hoped that further study of the life cycles of pest species of cecid would shed some light on the extraordinary ability of these species to reach pest status so rapidly and become such a serious economic pest of mushroom crops. Two species were studied: *Heteropeza pygmaea* and *Mycophila speyeri*. Three lines of *M. speyeri* were used during the study: two that had been maintained in laboratory culture for some time and one which had been isolated from an infestation at a local mushroom farm.

All lines of both species had the ability to reproduce paedogenetically. If culture conditions were maintained at an optimum then the cycle continued indefinitely. It was found that if culture conditions deteriorated (i.e. if the plates aged) the larvae could produce offspring which were destined to pupate and produce adult flies. All of the adult flies examined during this study were females. All of the adults studied more closely either laid eggs following eclosion or contained eggs on dissection. However, no eggs were seen to develop once laid and none gave rise to larvae.

It was initially presumed that the lines of cecid studied had become reproductively isolated after prolonged laboratory culture and had subsequently lost the ability to produce males, and therefore to reproduce in the adult stage. It was then found that the population which had been isolated from the mushroom farm behaved in the same way. It is concluded that many lines of these cecid species are functionally sterile as adults, and can reproduce only by the paedogenetic route.

It is true to say that although the production of adults may be seen as an important stage in the life cycle of cecids in terms of dispersion, the paedogenetic offspring themselves can fulfil a dispersive role. They have spines along their ventral side to aid in locomotion. These 'creeping welts' lead to the larvae appearing to be sticky; they are known to spread around a mushroom farm by sticking to worker's clothes and being carried from house to house. It is possible, that due to the great reproductive success of the paedogenetic cycle and the ability of the cecid larvae to be able to disperse themselves, cecid species have indeed lost the ability to produce male individuals and to reproduce sexually as adults.

The adult stage of these insects is not as damaging to mushroom crops; it does not stick to the fruiting bodies and cause cosmetic damage. If this stage is unable to give rise to offspring and therefore further generations, artificially diverging development to the adult form offers an avenue for possible pest control strategies.

Experiments were undertaken to try to elucidate the factor which causes a switch in the life cycle from one of paedogenetic reproduction to one of pupation and adult

production. This had been seen during routine culture when individual culture plates had become 'old'. An attempt was made to recreate these conditions by culturing the cecid lines on plates with different nutritional levels, seeding older fungal cultures with insects and by varying population densities of cecids. No one factor appeared to trigger the switch from paedogenetic reproduction and it was concluded that a combination of factors present in 'older' cultures may act to cause the switch.

Experiments were undertaken to establish whether a hormonal switch may be used to make paedogenetically reproducing cecid larvae produce adults. Went (1978a) had suggested that egg follicles were affected by exogenous hormone application when in *in vitro* culture. A number of hormonal insect growth regulating pesticides were used including ecdysteroid and juvenile hormone (JH) agonists. Although initial results suggested that a JH agonist may reduce the ability of these insects to reproduce, the results could not easily be replicated. It was concluded that due to their thick cuticles and apparent lack of response to these hormone analogues that paedogenetic cecids may not be good targets for control by hormonal growth regulators.

Insects synthesise their own ecdysteroid hormones (Rees and Isaacs 1985, Rees 1988). They utilise the steroid nucleus from the dietary sterols that they consume. Most insects use cholesterol as the starting point. It was found that the only sterol present in the diet of the mycetophagous species used in this study was ergosterol. This was interesting since a number of other mycetophagous insects have been shown to utilise unusual ecdysteroids because they were unable to dealkylate the more complex side chain of ergosterol (e.g. Ritter *et al.* 1982). If there was no cholesterol present in the

diet of these insects it was presumed that either they must dealkylate the ergosterol in their diet (in a similar fashion to insects that dealkylate phytosterols such as sitosterol), or that they must use the ergosterol in their diet to produce an alternative ecdysteroid to ecdysone such as makisterone (as do some other mycetophagous insects studied). Analysis of *H. pygmaea* tissues showed that this species did indeed dealkylate ergosterol, so that 50% of its body sterol was in the form of a cholestatriene. This would enable *H. pygmaea* to produce ecdysone and 20-hydroxyecdysone rather than using other, less conventional ecdysteroids. Analysis of the hormones present within the tissue confirmed the presence of 20-hydroxyecdysone and the absence of makisterone A.

A fascinating issue is how the paedogenetically developing embryos are nourished. This question was studied in depth. Maternal fat body is presumed to be the principal source of larval nutrients. Ultrastructural studies were performed on the fat body cells of paedogenetic larvae and the proteins contained within different regions of the insects were analysed using SDS-PAGE.

The ultrastructural study showed that the fat body cells of *M. speyeri* were multifunctional and that their structure changed as the insect developed. Strong evidence was found for protein synthesis by maternal fat body and although there was no indication of the active export of protein from the cells, it is possible that proteins may be stored in the fat body cells conjugated with lipids within the many lipid droplets found there.

No evidence was found for cell death in mother larval fat body cells from later stages in which oogenesis and offspring development had been completed. This is not the case with the fat body cells of insects which produce eggs as adults. It may be that although paedogenetic cecids have produced offspring they are still in the larval condition, and this may preclude the initiation of an apoptotic programme.

Authors such as Ivanova-Kasas (1965) have suggested that the transfer of nutrients from the maternal fat body to the offspring is a result of active consumption of the maternal tissues by the offspring or by the action of enzymes produced by the offspring on the fat body cells. The study of proteins present in the maternal fat body, maternal haemolymph and growing embryos showed that some fat body proteins did appear in the other tissues. *In vitro* culture of fat body cells succeeded in measuring the transfer of proteins from the fat body to the culture medium. The pattern produced appeared to be typical of that of haemolymph proteins, but the amount of protein released did not continue to increase with culture time. This suggests that the embryos growing within the maternal cuticle absorb nutrients, which have been exported by the fat body cells, from the surrounding haemolymph rather that directly from the fat body. In summary, evidence has been gathered for an active transfer of nutrients from maternal to embryonic tissues, and no evidence has been found for the parasitic or predatory "consumption" of the mother by her offspring.

Appendix 1.

Leatham's Medium.

Composition	**gl^{-1}**
D-Glucose	11.71
L-Glutamic acid	2.5
D-Glucoronic acid	4.0
KH_2PO_4	2.0
$MgSO_4.H_2O$	2.0
Mineral Solution	10 ml
Trace Element Solution	1 ml
Vitamin Solution	1 ml

Mineral Solution

$CaCl_2.\ 2H_2O$	3.67
$MnSO_4.5H_2O$	4.39
$ZnSO_4.7H_2O$	2.20

Trace Element Solution

Fe $(NH_4)_2(SO4)_2.6H_2O$	14.1
$CuSO_4.5H_2O$	0.784
$CaCl_2.6H_2O$	0.081
$Na_2MoO_4.2H_2O$	0.051
$NiCl_2.6H_2O$	0.081
$SnCl_2.2H_2O$	0.038
Conc. HCl	2 ml

Vitamin Solution	mgl^{-1}
(inactive) Inisitol	1000

Thiamine HCl	1000
Pyridoxine HCl	100
Nicotinic Acid	100
Na or Ca Pantothenate	100
p-aminobenzoic acid	100
Riboflavin	100
Biotin	30
Folic acid	10
Cyanocobalamin	10

Make up to 1 l with distilled water using 25 gl^{-1} N° 2 agar. Adjust to pH 5.7 with Conc. NaOH. This gives a C:N ratio of 35:1.

Appendix 2.

Solutions for Electron Microscopy Preparation.

Phosphate buffer.
Solution 1: Dibasic sodium phosphate (BDH) 0.2M
Na_2HPO_4. $2H_2O$ 35.61g in 1L

Solution 2: Monobasic sodium phosphate (BDH) 0.2M
NaH_2PO_4. $2H_2O$ 31.21g in 1L

500 ml of Solution 2 - 17.80 g
100 ml of Solution 1 - 3.121 g

For pH 7.45 combine in the following proportions 405 ml Solution 2 and 95 ml Solution 1

Washing Buffer.
0.1M Sörensen's buffer with 4% sucrose (4 g in 100 ml).

Paraformaldehyde.
Mix 5 g PFA powder into 50 ml of distilled water and while stirring heat to 60 - 65^{o}C.
Add drops of NaOH (Agar Scientific Ltd.) until the solution clears.
Allow to cool.

To make PFA, GDA Fixative (50 ml)
4 ml GDA, 10 ml PFA, 25 ml buffer, 10 ml distilled water.

Spurr's Resin.
ERL 4206 10 g
DER 736 6 g
NSA 26 g
Weigh, mix add S- 1, 0.4 g, drop-wise. Mix, Polymerise at 70 ^{o}C for 8 hours.

Appendix 3.

Lowry protein assay for microplates.

Solutions

Protein Standards.

400, 200, 100, 50, 25, 12.5, 6.25 μgml^{-1} Bovine Serum Albumen (BSA) (Sigma) in 0.25M sucrose made up from a stock BSA solution (10 $mgml^{-1}$ in water, OD^{280} 6.6).

Alkaline Copper Working Reagent

1 Volume of 0.5% w/v copper sulphate pentahydrate

1 Volume of 2.7% w/v sodium potassium tartrate

10 Volumes of 10 % w/v anhydrous sodium carbonate in 0.5M sodium hydroxide.

Folin-Ciocalteu reagent (Sigma), stock reagent diluted 10 X with water.

Method

50 µl each diluted sample into adjacent wells on a microplate. 50 µl sucrose solution into four wells as blanks.

50 µl of each BSA solution into two adjacent wells on the plate for constructing standard curve.

50 µl alkaline-copper working reagent into all wells containing samples, standards or blanks. Incubate at 30 °C for 20 minutes.

Add 100 µl Folin-Ciocalteu (working strength) to all wells. Incubate at 30 °C for 15 minutes.

Measure absorbance on plate reader at 620 nm.

Appendix 4.

Sodium dodecylsulphate polyacrylamide gel electrohporesis. (SDS-PAGE).

Loading Buffer	2% SDS	
	10% glycerol	
	0.001% bromophenol blue	
	5% (w/v) β mercaptoethanol	
	0.0625M Tris-HCl ph 6.8	
Stacking Buffer	0.5M Tris-HCl pH 6.8	
Resolving Buffer	1.5M Tris-HCl pH 8.8	
Running Buffer	3% Tris	
	14% glycine	
	1% SDS	
Resolving Gel (10%)	30 % acrylamide solution (BioRad)	3.25 ml
	resolving buffer	2.5 ml
	mQ water	4.2 ml
	ammonium persulphate (10%)	100 µl
	SDS (10%)	75 µl
	TEMED	10 µl
Stacking Gel (4.5%)	30% acrylamide solution (BioRad)	750 µl
	stacking buffer	1250 µl
	mQ water	3000 µl
	ammonium persulphate (10%)	50 µl
	SDS (10%)	50 µl
	TEMED	5 µl

Appendix 5.

Radioimmunoassay (RIA) of free ecdysteroids.

Borate Buffer

(0.1 M, pH 8.4)

Dissolve; 4.38 g sodium chloride (BDH),
6.21 g boric acid (Sigma),
9.53 g sodium tetraborate

In 1 l of distilled water and adjust pH.

H-22 Antisera

Produced from immunisation of rabbits with ecdysone-22-succinylthyroglobulin. Prepared in 5% (v/v) heat inactivated rabbit serum (I.R.S.) (Sigma) in borate buffer (100 µl).

Diluted in 11 ml 5% I.R.S. in borate buffer for use.

Labelled Ecdysone

[23, 24 (n) - ^{3}H] ecdysone (3.06 Tbq/ mmol; Nen, U.S.A.). Stock solution in toluene/methanol. Enough label was prepared for around 8000 c.p.m. per tube (100 tubes, 50 µl per tube) was prepared in 5% (v/v) I.R.S. in borate buffer. The toluene/methanol was blown off under nitrogen.

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*Unseen, from Matuszewski 1982.